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Oy Jalo Ant-Wuorinen Ab

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Patenttihakemus nro: 19992085  
Luokka: C12N X  
Hakija: Galilaeus Oy  
Asiamies: Oy Jalo Ant-Wuorinen Ab  
Asiamiehen viite: 31344

Määräpäivä: 02.11.2000 PW + TAVL

Patenttihakemuksen numero ja luokka on mainittava kirjelmässänne PRH:lle

Tarkastelun kohteena ovat olleet hakemuksenne selitysosaa ja patenttivaatimukset, jotka on toimitettu 29.9.1999.

Suoritetussa patentoitavuustutkimuksessa ei ole tullut esille patentoitavuuden esteitä. Tunnettua tekniikan tasoa kuvaavina julkaisuina esitetään julkaisut:

WPI An: 1996-209321, tiivistelmä julkaisusta WO-A-9610581, C07K 14/36, Galilaeus Oy.  
WPI An: 1997-036800, tiivistelmä julkaisusta US-A-5843735, C12N 15/00, Lee et al.  
WPI An: 1992-349214, tiivistelmä julkaisusta WO-A-9216629, C12N 15/31, Leiras Oy.

Patenttivaatimuksia ei voida hyväksyä niiden nykyisessä muodossa seuraavista syistä johtuen.

Patenttivaatimukset 1 ja 3 ovat epätasomallisia (PL 8 § 2 mom., PM 5 § kohta 2). Tuotteeseen kohdistetussa vaatimuksessa tulisi tuote kuvata rakenteen perusteella, jos rakenne tunnetaan ja/tai viittaamalla mikro-organismitalletukseen, jos tuote on talletettu.

Vaatimuksessa 3 haetaan tuotesuojaa DNA-fragmentille tai sen osalle. Vaatimuksen 3 ilmaisu -or a part thereof ei määrittele tuotetta riittävän selkeästi. Vaatimuksesta 3 tulisi käydä täsmällisemmin ilmi, mistä osista on kysymys. Myös ilmaisun -or a part thereof määrittelemien DNA-jaksojen tulee olla teollisesti käyttökelpoisia ja niiden tulee perustua samoihin keksinnöllisiin piirteisiin kuin on vaatimusten 1-2 DNA-fragmentilla (ks. PL 10 §, PA 16 §).

Käyttövaatimus 6 on esitetty paljon laajemmassa muodossa kuin hakemuksen selityksen nojalla on perusteltavissa, sillä selityksessä kuvattu DNA:n teollinen käyttö on käyttö aklasinomysiinin tuotteen lisäämiseksi (vrt. vaatimus 7), (PL 8 § 2 mom., PM 5 § kohta 2).

Vaatimuksen 12 ilmaisu metabolites on epätasomallinen. Tätä ilmaisua tulisi täsmennää, jotta vaatimuksesta 12 kävisi selkeämmin ilmi yhteys tuotetun metaboliitin ja siirrettyyn DNA-fragmentin välillä (PL 8 § 2 mom.).

Teitä pyydetään toimittamaan vastineenne yhteydessä uudet patenttivaatimukset, jotka on laadittu ottaen huomioon edellä mainitut seikat.

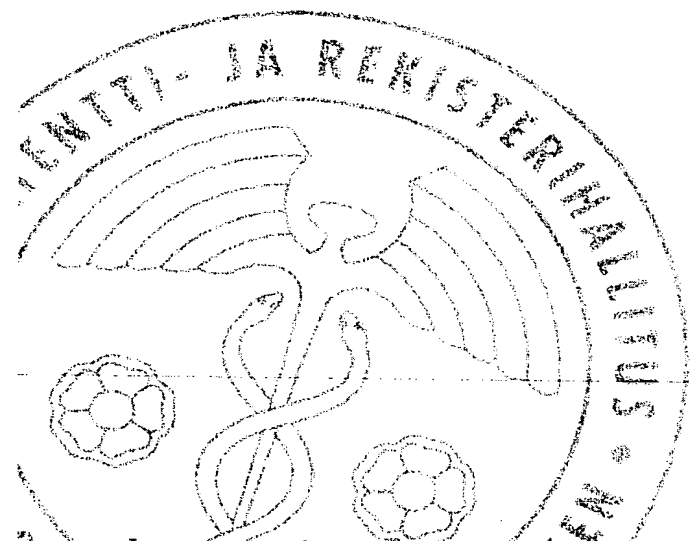
Teitä pyydetään toimittamaan myös hakemuksenne selityksen ja patenttivaatimusten suomen- tai ruotsinkielinen käännös. Ensiksi annettu suomen- tai ruotsinkielinen selitys ja patenttivaatimukset on hakemuksenne perusasiakirja (PA 21 §). Niiden sisällön tulee selvästi vastata virastossa olevien englanninkielisten asiakirjojen sisältöä (PM 38 a § 4 mom.).

Käännöksen tulee olla virastossa ennen kuin hakemus PL 22 §:n mukaan tulee julkiseksi ja sen mukana tulee olla hakijan tai hänen asiamiehensä vakuutus, että käännös vastaa alkuperäisiä asiakirjoja (PM 38 a § 3 mom).

*Pirkko*

Tutkijainsinööri  
Puhelin: (09) 69395348

Pirkko Karinen  
Bioteknologian tutkijat



Lausumanne huomautusten johdosta on annettava viimeistään yllämainittuna määräpäivänä. Jollette ole antanut lausumanne virastoon viimeistään mainittuna määräpäivänä tai ryhtynyt toimenpiteisiin tässä välipäätöksessä esitettyjen puutteellisuuden korjaamiseksi, jätetään hakemus sillensä (patenttilain 15 §). Sillensä jätetty hakemus otetaan uudelleen käsiteltäväksi, jos Te neljän kuukauden kuluessa määräpäivästä annatte lausumanne tai ryhdytte toimenpiteisiin esitettyjen puutteellisuuden korjaamiseksi ja samassa ajassa suoritate vahvistetun maksun, 320 mk hakemuksen ottamisesta uudelleen käsiteltäväksi. Jos lausumanne on annettu virastoon oikeassa ajassa, mutta esitettyjä puutteellisuuden ei ole siten korjattu, että hakemus voitaisiin hyväksyä, se hylätään, mikäli virastolla ei ole aihetta antaa Teille uutta välipäätöstä (patenttilain 16 §). Uusi keksinnön selitys, siihen tehdyt lisäykset ja uudet patenttivaatimukset on aina jätettävä kahtena kappaleena ja tällöin on otettava huomioon patenttiasetuksen 19 §.

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800015-47908

2/2 - (C) WPI / DERWENT  
AN - 1996-209321 [21]  
AP - WO1995FI00537 19950929; US19970809740 19970327; [Based on WO9610581 ]  
; WO1995FI00537 19950929; AU19950036103 19950929; [Based on WO9610581  
] ; WO1995FI00537 19950929; FI19970001308 19970327; EP19950933446  
19950929; WO1995FI00537 19950929; [Based on WO9610581 ] ;  
WO1995FI00537 19950929; JP19960511427 19950929; [Based on WO9610581 ]  
PR - FI19940004556 19940930  
TI - New DNA contg. genes involved in anthracycline biosynthesis - from  
Streptomyces nogalater, used to produce anthracycline cpds. and their  
precursors, including new cpds. in other Streptomyces  
IW - NEW DNA CONTAIN GENE ANTHRACYCLINE BIOSYNTHESIS STREPTOMYCES PRODUCE  
ANTHRACYCLINE COMPOUND PRECURSOR NEW COMPOUND STREPTOMYCES  
IN - HAKALA J; MANTSALA P; YLIHONKO K; MAENTSAELAE P  
PA - (GALI-N) GALILAEUS OY  
PN - US5986077 A 19991116 DW200001 C07H21/04 000pp  
- WO9610581 A1 19960411 DW199621 C07K14/36 Eng 058pp  
- AU3610395 A 19960426 DW199631 C07K14/36 000pp  
- FI9701308 A 19970327 DW199725 C07K0/00 000pp  
- EP0792285 A1 19970903 DW199740 C07K14/36 Eng 000pp  
- JP10506533T T 19980630 DW199836 C12N15/09 059pp  
ORD - 1996-04-11  
IC - C07H21/04 ; C07K0/00 ; C07K14/36 ; C12N15/09 ; C12N15/31 ; C12N15/76 ;  
C12P1/04 ; C12P15/00 ; C12P19/56  
FS - CPI  
DC - B05 D16  
DS - AT BE CH DE DK ES FR GB GR IE IT LU MC NL PT SE LI  
DN - AU CA CZ FI HU JP KR NO RU US  
CT - 2.Jnl.Ref; WO9216629  
AB - WO9610581 New isolated and purified DNA fragment (A) is a gene  
fragment of the anthracycline biosynthesis pathway of Streptomyces  
nogalater and is present in the actI-hybridising 12kb BglII fragment  
of the S.nogalater genome. Also new are (1) recombinant DNA construct  
contg. (A); and (2) the new anthracycline precursor of formula (I).  
- USE - (A) is used, after transfer into a foreign Streptomyces strain,  
for prodn. of anthracyclines and their precursors, esp. auramycinone  
and its glycoside (useful as cytostatic agents).  
- ADVANTAGE - (A) makes possible prodn. of hybrid anthracyclines and  
precursors (including new cpds.), even in Streptomyces that do not  
normally produce them.  
- (Dwg.3/7)  
USAB- US5986077 New isolated and purified DNA fragment (A) is a gene  
fragment of the anthracycline biosynthesis pathway of Streptomyces  
nogalater and is present in the actI-hybridising 12kb BglII fragment  
of the S.nogalater genome. Also new are (1) recombinant DNA construct  
contg. (A); and (2) the new anthracycline precursor of formula (I).  
- USE - (A) is used, after transfer into a foreign Streptomyces strain,  
for prodn. of anthracyclines and their precursors, esp. auramycinone  
and its glycoside (useful as cytostatic agents).  
- ADVANTAGE - (A) makes possible prodn. of hybrid anthracyclines and  
precursors (including new cpds.), even in Streptomyces that do not  
normally produce them.

1/2 - (C) WPI / DERWENT

AN - 1997-036800 [04]

AP - JP19950139986 19950515 JP19950139986 19950515; [Previous Publ.  
J08214886 ] US19950551211 19951031

PR - KR19950001950 19950203

TI - DNA encoding aclavinone C-11 hydroxylase - useful for prodn. of new  
hybrid anthracycline antibiotic, designated aclacinomycin X

IW - DNA ENCODE HYDROXYLASE USEFUL PRODUCE NEW HYBRID ANTHRACYCLINE  
ANTIBIOTIC DESIGNATED ACLACINOMYCIN

IN - HONG S; HONG Y; HWANG C; KIM H; KIM Y; LEE J

PA - (KORE-N) KOREA RES INST CHEM TECHNOLOGY

- (NITT-N) NITTO SEIYAKU KK

- (ILDO-N) IL-DONG PHARM CO LTD

- (KOAD ) KOREA ADV INST SCI & TECHNOLOGY

PN - JP8214886 A 19960827 DW199704 C12N15/09 020pp

- JP2731367B2 B2 19980325 DW199817 A61K31/71 019pp

- US5843735 A 19981201 DW199904 C12N15/00 000pp

ORD - 1996-08-27

IC - A61K31/71 ; C07H15/252 ; C07H21/04 ; C12N1/21 ; C12N9/02 ; C12N9/14 ;  
C12N15/00 ; C12N15/09 ; C12P19/60 ; C12P21/06

FS - CPI

DC - B04 D16

AB - J08214886 A gene (I) encoding aclavinone C-11 hydroxylase is claimed.

Also claimed are: (1) aclavinone C-11 hydroxylase having the 489 amino  
acid sequence (given in the specification); (2) a vector contg. (I);

(3) a host cell transformed by the above plasmid; and (4) an  
anthracycline type cpd. designated aclacinomycin X: (II).

- USE - The vector contg. (I) is useful, when expressed by a transformed  
host cell, for the prodn. of a hybrid anthracycline antibiotic which  
is absent in the wild type microbe.

- (Dwg.0/17)

USAB- US5843735 A gene (I) encoding aclavinone C-11 hydroxylase is claimed.

Also claimed are: (1) aclavinone C-11 hydroxylase having the 489 amino  
acid sequence (given in the specification); (2) a vector contg. (I);

(3) a host cell transformed by the above plasmid; and (4) an  
anthracycline type cpd. designated aclacinomycin X: (II).

- USE - The vector contg. (I) is useful, when expressed by a transformed  
host cell, for the prodn. of a hybrid anthracycline antibiotic which  
is absent in the wild type microbe.

2/2 - (C) WPI / DERWENT

AN - 1992-349214 [13]  
AP - WO1992FI00084 19920323; FI19910001441 19910325; AU19920014461 19920323;  
WO1992FI00084 19920323; [Based on WO9216629 ] ; FI19910001441  
19910325; [Previous Publ. FI9101441 ]  
PR - FI19910001441 19910325  
TI - Hybrid anthracycline antibiotic DNA from Streptomyces purpurascens -  
obtd. by hybridisation with S. galilaeus DNA, useful for prodn. of  
antibiotics difficult to produce by other means.  
IW - HYBRID ANTHRACYCLINE ANTIBIOTIC DNA STREPTOMYCES OBTAIN HYBRID  
GALILAEUS DNA USEFUL PRODUCE ANTIBIOTIC DIFFICULT PRODUCE  
IN - HAKALA J; KOPIO A; LAMPI K; NIEMI J; PAERSSINEN R; YLIHONKO K  
PA - (LEIR-N) LEIRAS OY  
PN - WO9216629 A1 19921001 DW199242 C12N15/31 Eng 053pp  
- FI9101441 A 19920926 DW199251 C12P19/56 000pp  
- AU1446192 A 19921021 DW199303 C12N15/31 000pp  
- FI93860B B 19950228 DW199513 C12P19/56 000pp  
ORD - 1992-09-26  
IC - C07H15/252 ; C12N1/21 ; C12N15/11 ; C12N15/31 ; C12N15/76 ; C12P19/56  
FS - CPI  
DC - B05 D16  
DS - AT BE CH DE DK ES FR GB GR IT LU MC NL SE  
DN - AU CA HU JP KR NO RU US  
CT - 4.Jnl.Ref; EP0131181; EP0298385; EP0299350; EP0050724  
AB - WO9216629 Sequence (I) has been isolated by:- (a) isolating a DNA  
fragment that hybridises with the actI0.8 probe from S. galilaeus; and  
(b) using the fragment in the hybridisation of S. purpurascens. When  
transferred to a S. galilaeus host, which naturally produces  
glycosides of aklavinone, produces anthracyclines not naturally  
synthesised. Recombinant DNA construct comprises a vector contg. (I),  
amplified in micro-organisms of the genus Streptomyces; Streptomyces  
strain contains construct prodn. of hybrid antibiotics of the  
anthracycline gp. comprises growing strain under pref. conditions and  
sepg. antibiotics from the growth medium and purifying.  
- USE/ADVANTAGE - Developed for opt. clinical use(Dwg.1/9)



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**(B) (11) KUULUTUSJULKAISU  
UTLAGGNINGSSKRIFT**

93860

**(15) Patentti myönnetty  
Patent meddelat 12 06 1990**

(51) Kv.1k.6 - Int.cl.6

C 12P 19/56, C 12N 15/11, 15/76, 1/21, C 07H 15/252

**SUOMI-FINLAND****(FI)****Patentti- ja rekisterihallitus  
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(74) Asiamies - Ombud: Oy Jalo Ant-Vuorinen Ab

(54) Keksinnön nimitys - Uppfinningens benämning

Menetelmä antibioottien tuottamiseksi, siinä käyttökelpoisia DNA-jaksoja,  
yhdistelmä-DNA-konstruktioita ja näitä sisältäviä mikrobikantoja  
Förfarande för att producera antibiotika, däri användbara DNA-sekvenser,  
hybrid-DNA-konstruktioner och dessa innehållande mikroorganismer

(83) Mikro-organismitalletus - Deposition av mikroorganism: 6403 DSH  
6404 DSH

(56) Viitejulkaisut - Anförda publikationer

DD C 284694 (C 12N 15/76), US A 3988315 (C 07H 15/24), US A 4375511 (C 12N 1/00),  
US A 4209588 (C 12P 19/56),  
J. Bacteriology 172 (9) (1990) 4816-4826 (Bartel et al.),  
Proc. Natl. Acad. Sci. USA 84 (1987) 4445-4449 (Motamedi et al.),  
Abstr. Annu. Meet. Am. Soc. Microbiol. 1988 tiivistelmä 215 (Bartel et al.),  
Nature 314 (1985) 642-644 (Hopwood et al.), Nature 325 (1987) 818-821 (Malpartida et al.),  
Proc. Natl. Acad. Sci. USA 86 (1989) 3135-3139 (Stutzman-Engwall et al.)

(57) Tiivistelmä - Sammandrag

Keksinnön kohteena on menetelmä, jossa tunnettujen *Streptomyces*-suvun mikro-organismien avulla, siirtämällä niihin määrättyjä geenejä tietyistä, rakenteeltaan lähisukuisia antibiootteja tuottavista mikrobikannoista, tuotetaan bioteknisesti sellaisia antrasykliiniryhmän antibiootteja, joita nämä mikro-organismit eivät luontaisesti tuota. Keksinnön kohteena ovat myös tällaiseen menetelmään tarvittavat yhdistelmä-DNA-tekniikalla muodostetut mikro-organismit, yhdistelmä-DNA-konstruktiot ja niissä tarvittavat DNA-jaksot. Keksintö sijoittuu antibioottien bioteknistä tuottoa käsittelevälle alalle, ja koskee hybridiantibioottitekniikan soveltamista antrasykliiniryhmän antibiootteihin.



(51) International Patent Classification <sup>5</sup> :  
C12N 15/31, C12P 19/56

A1

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(30) Priority data:  
911441 25 March 1991 (25.03.91) FI

(71) Applicant (for all designated States except US): LEIRAS OY  
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(72) Inventors; and

(75) Inventors/Applicants (for US only): NIEMI, Jarmo [FI/FI];  
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KOPIO, Anja [FI/FI]; Tähkäpolku 2 B 37, SF-20720  
Turku (FI). PÄRSSINEN, Raimo [FI/FI]; Kappeliranta,  
SF-21610 Kirjala (FI). YLIHONKO, Kristiina [FI/FI];  
P. Katariinantie 8 B 19, SF-20780 Kaarina (FI). LAMPL,  
Klaus [FI/FI]; Rusthollinkatu 4 I, SF-20880 Turku (FI).

(74) Agent: OY JALO ANT-WUORINEN AB; Laivuririnne  
2 A, SF-00120 Helsinki (FI).

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tent), FR (European patent), GB (European patent), GR  
(European patent), HU, IT (European patent), JP, KR,  
LU (European patent), MC (European patent), NL (Eu-  
ropean patent), NO, RU, SE (European patent), US.

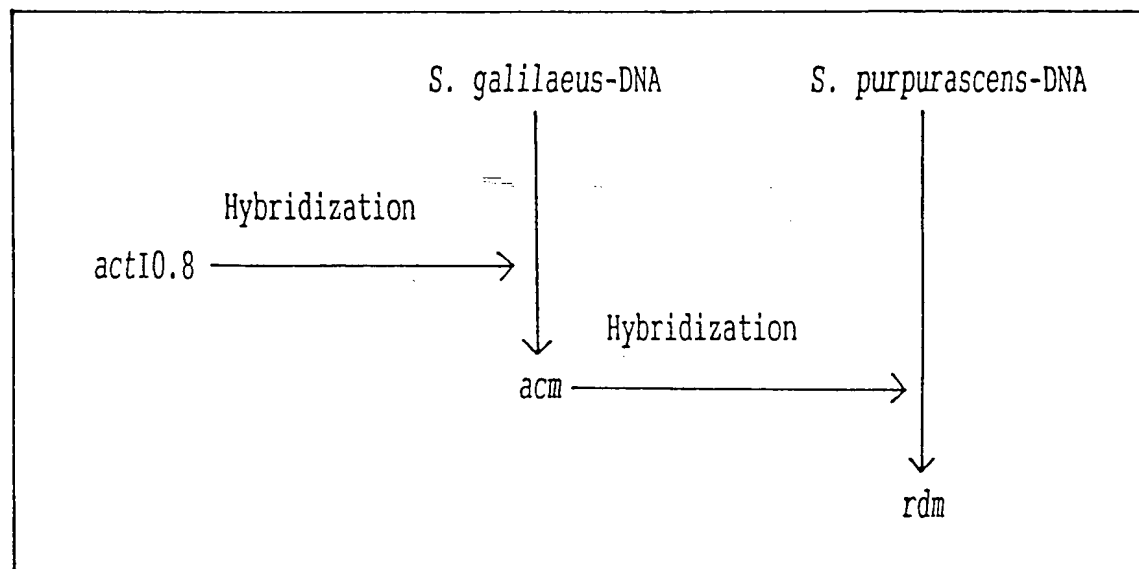
Published

With international search report.

With amended claims.

In English translation (filed in Finnish).

(54) Title: PROCESS FOR THE PRODUCTION OF ANTIBIOTICS, DNA-SEQUENCES, RECOMBINANT-DNA-CON-  
STRUCTS AND MICROBIAL STRAINS USED THEREIN



(57) Abstract

The invention concerns a process according to which by means of known microorganisms of the *Streptomyces*-genus, by transferring into them specific genes from certain microbial strains which produce structurally closely related antibiotics, such antibiotics of the anthracycline group are biotechnically produced, which these microorganisms do not produce naturally. The invention concerns also microorganisms needed in such a process and formed by recombinant-DNA-technology, recombinant-DNA-constructs and DNA-sequences needed therein. The invention belongs to the field of biotechnical production of antibiotics and relates to the application of hybrid antibiotic technology to antibiotics of the anthracycline group.



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ES	Spain				

Process for the production of antibiotics, DNA-sequences, recombinant-DNA-constructs and microbial strains used therein

5

The present invention concerns a process by which, by means of known microorganisms, by transferring into them specific genes from certain microbial strains which produce structurally closely related antibiotics, such  
10 antibiotics of the anthracycline group are biotechnically produced, which these microorganisms do not produce naturally. The invention concerns also microorganisms needed in such a process and formed by recombinant-DNA-technology, recombinant-DNA-constructions and DNA-sequences  
15 needed therein. The invention belongs to the field of biotechnical production of antibiotics and relates to the application of hybrid antibiotic technology to antibiotics of the anthracycline group.

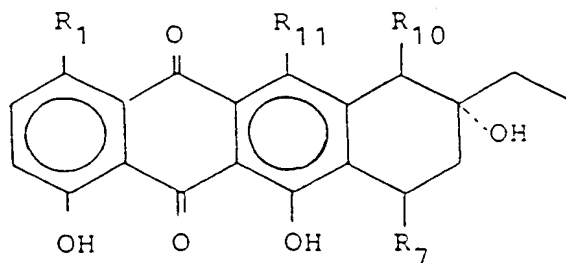
20

Molecules, which in the same molecule have structural features from two such antibiotics which are not produced naturally by one microorganism, are called hybrid antibiotics. Such molecules can in principle, and in some cases also in practice be produced by biotransformation,  
25 i.e. by giving an antibiotic, produced by one microorganism, to another microbe which converts the molecule. The use of the term is, however, established to mean the fact that biosynthetic genes of one antibiotic are transferred by recombinant-DNA-technology into a microbe producing  
30 another antibiotic, and thus the latter microbe is made to produce antibiotics, which it does not itself, nor the gene donor strain produce naturally. The hybrid antibiotics technique is described e.g. in H. G. Floss (Trends in Biotechnology, Vol. 5, 1987, pages 111-115),  
35 "Hybrid antibiotics - the contribution of the new gene combinations" and in the references given therein.

An antibiotic molecule is formed in a microorganism, which produces it by an enzymatic reaction cascade including typically from 10 to 20 enzymes. The first enzymes in the chain use as their substrates the normal intermediates of the cell metabolism, but as the molecule proceeds in the reaction chain, usually various rather exotic structural features are formed therein, when considering the matter on the basis of the so called primary metabolism. An important characteristic from the point of view of obtaining hybrid antibiotics is the fact that these enzymes are believed to have relatively minor substrate specificity, i.e. that they are able to use as their substrate also compounds which differ as to their structure from those which are found in the original microbe.

Another important characteristic, which has been shown in the microbial genus *Streptomyces* by the studies of the biosynthetic genetics of antibiotics, is the fact that the biosynthetic genes of an antibiotic are clustered which means that they exist in the microbial DNA close to each other. This has in many cases made it possible to isolate other genes, which participate in the biosynthesis of the antibiotic, one gene participating in the biosynthesis having been identified by some procedure.

The anthracyclines are a broad group of compounds having the common skeletal structure of 7,8,9,10-tetrahydro-5,12-naphthacene quinone of the general formula I



In typical anthracyclines, various substituents are linked to this skeletal structure, the most important group of which are formed by some sugar derivatives. Several of the substances of the anthracycline group are in use as cytostatic drugs in the treatment of cancer, such as e.g. daunorubicin, doxorubicin and aclarubicin. Antibiotics of the anthracycline group are presented e.g. in the article of A. Fujiwara and T. Hoshino: "Anthracycline antibiotics" (CRC Critical Reviews in Biotechnology, vol. 3, 1986, pages 133-157) and in the references cited therein. The anthracyclines belong to the antibiotics with a so-called polyketide structure.

The relatively complex structure of the anthracyclines has retarded the development of novel compounds with better characteristics. It has been possible to prepare synthetically a large group of anthracyclines, but screening of microorganisms belonging mainly to the genus *Streptomyces* from the soil has also formed an important source of new anthracyclines. This procedure is not satisfactory, because it does not make it possible to convert the anthracycline structure systematically, but discovery of new anthracyclines is random.

The principle of hybrid antibiotics seems to make it possible to convert the anthracycline structure systematically. Despite of the fact that the first hybrid antibiotics were described in 1985 (Hopwood et al., 1985b), only few successful experiments for obtaining hybrid antibiotics have been described so far. Hutchinson et al. (1989) list four publications wherein a successful process for producing hybrid antibiotics is described. They show several facts which may prevent the obtaining of hybrid antibiotics: Some hosts cannot possibly be transformed with foreign DNA because of restriction or expression bars, foreign genes may, due to the control

sequences which they comprise, prevent the host from producing antibiotics, the hybrid antibiotic produced may be toxic to the host, and the expression of transferred genes may demand control factors, which maybe are not present in the host. Hereto can be added the general problem that the genes, which in the donor participate in the biosynthesis of the antibiotics, are to be identified and distinguished from other genes. Finally, the substrate specificity of the enzymes, the encoding genes of which are transferred, may, however, be so strict that the substrates provided by the host are not converted to new compounds.

Consequently, although it can be anticipated, that by transferring genes participating in the biosynthesis from one *Streptomyces* to another, new compounds could be obtained, it cannot be concluded in advance, what is obtained and if the experiment is successful in general. Thus, the identification of such a gene sequence and the construction of a recombinant-DNA-construction with which, by using a suitable host, hybrid antibiotics are obtained successfully, forms a significant and industrially useful invention.

We have now invented a process according to which certain microorganisms of the genus *Streptomyces* producing anthracyclines are made to produce anthracycline antibiotics new to them. We have transferred into them DNA-sequences which can be identified according to the manner described hereafter and which originate from rhodomycin producing microorganisms belonging to the species *Streptomyces purpurascens*, as a suitable recombinant-DNA-construction and using recombinant-DNA-technology.

Our aim to find DNA-sequences with which hybrid antibiotics of the anthracycline group could be obtained was based on the finding of Malpartida et al. (1987) that the

biosynthetic genes of antibiotics having the polyketide structure have significant similarities, which may make it possible to isolate biosynthetic genes of other polyketide antibiotics, when a certain probe of biosynthetic genes of one polyketide antibiotic is available, comprising a DNA-sequence which is similar between different species, which probe is used in DNA hybridization techniques. However, by this procedure biosynthetic genes cannot be found reliably, but e.g. Stutzman-Engwall and Hutchinson (1989) found by this procedure from *S. peuce-*  
10 *tius* five different gene regions containing domains homological to actI probe: from these only one has been shown to include the biosynthetic genes of doxorubicin. It is to be noted that the acm probe described hereafter  
15 identifies from *S. peucetius* exactly this gene region of five possible regions.

On the basis of the published data it is characteristic to the biosynthesis of different anthracyclines that in  
20 the biosynthesis first an anthracycline aglycone called aklavinone is formed, wherefrom other anthracyclines are formed as a result of the activity of various modifying enzymes. Consequently, we came to the conclusion that a suitable host microbe, after transfer into which the  
25 expression of these modifying enzymes could be detected, would be a *Streptomyces* strain producing aklavinone glycosides. Suitable microbe strains proved to be *Streptomyces galilaeus* ATCC 31615 and ATCC 31133.

30 When selecting the microbial strain, wherefrom we would try to transfer the genes of the modifying enzymes, we decided on the *Streptomyces purpurascens* microbial species, because it is supposed to have several enzyme activities which modify the aglycone moiety, namely the  
35 activities modifying the positions 1, 10 and 11, and especially modifications concerning the position 10

seemed to be lacking among the known aglycones. Consequently, the gene donor strain was selected to be *S. purpurascens* ATCC 25489, which is a type strain of the species *S. purpurascens*.

5

The strategy, which led to the finding of the DNA sequences described in this application, was thus the following: A region homologous to the *actI* region described by Malpartida et al. is isolated from *S. purpurascens*, DNA sequences from the region surrounding it are  
10 transferred to *S. galilaeus*, the recombinant-DNA-strains so obtained are cultivated under conditions in which the host strain naturally produces an antibiotic, and the antibiotics produced are analyzed to show if any of the  
15 DNA sequences cause production of new compounds. As a probe the moiety of the above mentioned *actI* region was used, which is marked *actI0.8*.

When the *actI0.8* probe was hybridized to the *S. galilaeus*  
20 and *S. purpurascens* DNA transferred to membranes by the Southern technique, it was found that *S. galilaeus* DNA gave a distinct signal, which corresponded to a *Bam*HI fragment of ca 3 kb. *S. purpurascens* gave on the other hand a fairly weak signal, which corresponded to a *Bam*HI  
25 fragment of ca 8 kb. As difficulties in the cloning of genes can occur by weak cross hybridization, the biosynthetic genes of *S. purpurascens* were isolated by isolating first the DNA sequence corresponding to the *actI* from *S. galilaeus* and using then this as a probe,  
30 assuming that it would give a better hybridization signal as it is a biosynthetic fragment of a structurally closer antibiotic (Figure 1).

The region homologous with *actI0.8* isolated from *S.*  
35 *galilaeus*, which was marked *acm*, proved to be a very

useful probe when isolating at least some biosynthetic genes of anthracyclines. In addition to the genes described in this application which led to hybrid antibiotics, this probe recognized from *S. peucetius* specifically the biosynthetic genes of doxorubicin, which were described by Stutzman-Engwall and Hutchinson (1989).

When the Southern hybridization was repeated using the *acm* probe, *S. purpurascens* DNA was found to give a distinct hybridization signal, which corresponded to a *Bam*HI fragment of ca 8 kb. Around the DNA sequence giving this signal several DNA sequences were isolated, which were transferred into *S. galilaeus* by a procedure similar to that described hereafter in the experimental part. However, from the DNA sequences tested only the sequence according to the invention caused production of new compounds.

The strains according to the invention can be reproduced according to the following description; in addition, the reproducibility of the invention has been secured by depositing the crucial microbial strains and plasmids in a depository according to the Budapest treaty. It is obvious to a man skilled in the art that the process steps used in the recombinant DNA techniques are known as such, but the inventive step lies in the fact that these steps are carried out according to a specific strategy to give a new result. It is also obvious to a man skilled in the art that for carrying out individual steps, alternative processes have often been described, by which the steps given in this description can be replaced by using good professional skill.

The present invention thus relates to a process for producing hybrid antibiotics of the anthracycline group which process comprises



- isolating from a *Streptomyces purpurascens* strain producing anthracyclines a DNA sequence leading to the expression of hybrid antibiotics of the anthracycline group,  
5
- constructing a recombinant-DNA-construction comprising the said DNA sequence,
- transforming the recombinant-DNA-construction  
10 obtained to a *Streptomyces galilaeus* host producing aklavinone glycosides,
- cultivating the transformed strain obtained under conditions wherein the host strain  
15 naturally produces an antibiotic, and
- recovering the hybrid antibiotic as formed.

20 The present invention also relates to the said DNA sequences isolated from the bacterium *Streptomyces purpurascens*, which lead to the production of the hybrid antibiotics as described.

25 The said recombinant-DNA-construction can be constructed by ligating such a DNA sequence according to the invention to a suitable vector, which is preferably a vector amplifying in microorganisms of genus *Streptomyces*, e.g. the plasmid pIJ486 (Ward et al., 1986). When such a recombinant-DNA-construction is transformed to a *S. gali-*  
30 *laeus* host, preferably to a host, which produces aklavinone glycosides, especially to the above mentioned *S. galilaeus* strains, a *Streptomyces* strain producing antibiotics of the anthracycline group is obtained.

35 The compounds produced are new anthracycline antibiotics, for which cytostatic activity has been shown (Example

12). Thus the compounds of the present invention are interesting compounds to be further developed for possible clinical use. The production of the said compounds by some other process than the fermentation of a hybrid strain is very difficult.

In the following the isolation of the DNA sequences of the invention from the *S. purpurascens* strain ATCC 25489, the production of the hybrid antibiotics by the strain as obtained, and the purification and structural analysis of the aglycone moieties of the hybrid antibiotics as produced are given in detail as an example of the preferred embodiments of the invention. Detection of this DNA sequence leading to the hybrid production of ATCC 25489 can be made on the basis of the characteristics as described, but also from the fact that it comprises the following nucleotide sequence beginning from the fourth BamHI recognition site from the left in the restriction map (Figure 3):

```
1  GATCCCTATG CCAGAGCACC GTCAGCAACG GGCNCTCCGC ATGGGCGTGA
51  TCGGCACGGC GAACATCGCG ATTCGCCGGA TCATGCCCCGT GCNCNCCGCG
101 CATGACCACG TCGACCTTGT CGCGGTGGCC AGCCGGGACA AGGCCCGGGC
151 CGAGCGGGTG GGGGCCGCTT TCGGCTGCGG TGGCGTGGGG GATTACGCGG
201 CGCTCGTCGA GCGGACGACC TTGACNCGTC TATATTCCGC TGCCGCCCCG
251 CATGCATCAC GAGTGGGCGC TGCGGGCTTT GCGTTCGGGA AAGCACGTGC
301 TGGTCGAGAA ACCGATGTCT GACACGTACG AGAAACTCT CGAGCTGATG
351 TCGACCGCGT CGGAACTCGG ACTCGTGCTC GCCGAGAACT TCATGTTCTT
401 GCACCATTCC CAGCACGCNG CGGTACNCG ATGCTCGACG AGTCCGTGGG
451 AGAACTGCGG CTCTTCTCCN GNNCGTTCNC CGTNNGCCGC TGGCACCCGA
501 GTGTTCCGGT ACCAG
```

in which N refers to one or more nucleotides which have not been identified.

Brief description of the drawings

- 5
- Fig. 1      A schematic presentation of the procedure, by which the region homological to the conserved region actI of the biosynthetic genes of the polyketide antibiotics was recognized in *S. purpurascens*-DNA.
- 10
- Fig. 2      The restriction map of the plasmid pIJ2345.
- Fig. 3      The restriction map of the *rdm*-clones cloned from *S. purpurascens* and the part of the chromosome covered by them. The region recognized by the *acm*-probe is marked as shaded.
- 15
- Fig. 4      The restriction map of the plasmid pH2008.
- 20
- Fig. 5      The thin layer of the fractions obtained when fractioning the aglycon mixture.
- Fig. 6      The proton-NMR-spectrum and structure of Aglycon IV
- 25
- Fig. 7      The mass spectrum of Aglycon IV (chemical ionization, negative ions)
- Fig. 8      The proton-NMR-spectrum of the hybrid glycoside IVT
- 30
- Fig. 9      The mass spectrum of the hybrid glycoside IVT

Solutions and growth media used

	SGYEME	per litre
5		
	Yeast extract (Difco)	3 g
	Bacto-peptone (Difco)	5 g
	Malt extract (Oxoid)	3 g
	Glucose	10 g
10	Sucrose	100 g

Sterilize by autoclaving 20 min 121 °C. After autoclaving add 2 ml of sterile 2M MgCl<sub>2</sub> solution per litre and 50 ml of sterile 10% glycine solution per litre.

15

## LYSOZYME SOLUTION

	Sucrose	0.3 M
	Tris (tris-hydroxymethylaminomethane) pH 8	25 mM
20	EDTA (ethylenediaminetetraacetic acid) pH 8	25 mM
	Lysozyme (Sigma)	2 mg/ml

## PHENOL MIXTURE

25	Phenol (Ultrapure, Gibco BRL)	500 g
	8-hydroxyquinoline	0.5 g

Saturate by 50 mM Tris-HCl buffer, pH 8.

30 "2 \* KIRBY MIXTURE"

	Sodium-tri-isopropyl-naphthalenesulphonate (Fluka)	2 g
	Sodium-4-aminosalicylate	12 g
	2M Tris-HCl buffer pH 8	5 ml
35	Phenol mixture	6 ml
	H <sub>2</sub> O ad.	100 ml

12

## PHENOL-CHLOROFORM

	Phenol mixture	50 ml
	Chloroform	50 ml
5	Isoamyl alcohol	1 ml

## TE

	Tris-HCl buffer, pH 8.0	10 mM
10	EDTA, pH 8.0	1 mM

## SSC

15 Various dilutions are used, as e.g. 2 \* SSC, 6 \* SSC etc., which are prepared from the basic solution 20 \* SSC:

	20 * SSC	per litre
20	NaCl	175.3 g
	Na-citrate	88.2 g

Adjust pH to 7 with NaOH; sterilize by autoclaving.

25 DENHARDT'S SOLUTION (Maniatis et al., p. 448)

	Basic solution 50 *:	
	Ficoll® (Pharmacia)	5 g
	Polyvinyl pyrrolidone	5 g
30	Bovine serum albumin	5 g
	Distilled water ad.	500 ml

Filter through 0.45 µm sterile filter, divide to 5 ml aliquots and store in freezer -20 °C.

35

## E1 GROWTH MEDIUM

Per litre:

- 5
- |   |     |   |
|---|-----|---|
| Glucose   | 20  | g |
| Soluble starch  | 20  | g |
| Pharmamedia®  | 5   | g |
| Yeast extract   | 2.5 | g |
| 10 K <sub>2</sub> HPO <sub>4</sub> · 3 H <sub>2</sub> O | 1.3 | g |
| MgSO <sub>4</sub> · 7 H <sub>2</sub> O                  | 1   | g |
| NaCl  | 3   | g |
| CaCO <sub>3</sub>                                       | 3   | g |
- 15 Mix with tap water and adjust pH to 7.5 with NaOH. Sterilize by autoclaving.

EXAMPLE 1.Preparation of the probe

- 20 The probe actI described in the Malpartida et al. publication i.e. the 2.2 kb (kilobase, thousand base pairs, the unit of measurement of the molecule length of DNA) BamHI fragment derived from the actI region in the vector pBR329 = pIJ2345, was obtained from professor D. A.
- 25 Hopwood (John Innes Institute, Norwich, UK). A 0.8 kb BglII-fragment was used as the probe. The restriction map of the plasmid pIJ2345 is given in Fig. 2.
- 30 *E. coli* strain W 5445 carrying the plasmid pIJ2345 was grown, the plasmid was isolated therefrom by neutral SDS degradation (Maniatis et al., p. 92) and the plasmid was purified by centrifuging in a cesium chloride-ethidium bromide gradient (Hopwood et al. 1985a, p. 83, steps 17-21). The plasmid fraction from the gradient was ex-
- 35 tracted with isopropanol and precipitated with ethanol (Hopwood et al. 1985a, p. 127).

The 0.8 kb *Bgl*III fragment was isolated by digesting the pIJ2345-plasmid prepared above, by *Bgl*III endonuclease (Boehringer Mannheim or New England Biolabs) according to the instructions of the manufacturer, and by separating  
5 the fragment from the rest of the plasmid by preparative agarose gel electrophoresis (Hopwood et al. 1985a, p. 137). The fragment cut away from the gel was purified from the agarose using the GeneClean reagent kit (Bio 101) according to the instructions of the manufacturer.

10 About 100 ng of the isolated probe fragment was labelled with  $\alpha^{32}\text{P}$ -deoxyadenosine phosphate (New England Nuclear NEG-021H, 3000 Ci/mmol) for the hybridization, using the random prime labelling reagent kit of Boehringer Mannheim  
15 according to the instructions of the manufacturer. The labelled DNA was separated from the radioactive nucleotide in a Nick column (Pharmacia) according to the instructions of the manufacturer.

20 EXAMPLE 2.                      Isolation of the total DNA from the  
*Streptomyces* strains

The *Streptomyces* strains ATCC 31615 and ATCC 25489 were obtained from the American Type Culture Collection. In  
25 order to isolate the total DNA they were grown in 50 ml of SGYEME medium in 250 ml erlenmeyer flasks, which were shaken at 250 rpm at 28.5 °C for ca 50 hours.

The total DNA was isolated in the following manner, which  
30 is a modification of that described by Hopwood et al. (Hopwood et al. 1985a, p. 77).

The culture was separated by centrifuging for 10 min at ca 3000 g, resuspended in 3 ml of lysozyme solution and  
35 incubated for 10 min in a 37 °C water bath. 4 ml of "2 \*

Kirby mixture" reagent was added and stirred carefully by turning around the tube for ca 1 min.

8 ml of phenol-chloroform was added, stirred as above and centrifuged for 10 min at ca 3000 g. The upper water phase from the tube was transferred with a pipette with an open tip to another test tube, which contained 3 ml of phenol-chloroform and stirred and centrifuged as above. The water phase was transferred to another test tube with a pipette with an open tip, 3M sodium acetate, the pH of which had been adjusted to 6 with acetic acid, was added in the amount of 1/10 of the volume of the water phase, and the mixture was stirred. Thereafter an equal amount of isopropanol was carefully pipetted on the top of the water phase and the high molecular DNA, which precipitated at the interface of the water and isopropanol phases, was carefully "spinned" around a sterile glass rod. The glass rod with its DNA precipitate was transferred to another test tube, which contained 7 ml of 70% ethanol, the precipitate was released from the glass rod into an empty test tube and it was desiccated in a vacuum desiccator for 5-10 min. The DNA was dissolved in 1-2 ml of TE. The concentration and molecular size thereof was measured by running an agarose gel electrophoresis (Hopwood et al. 1985a, p. 136, buffer TAE, 0.3 - 0.6 % agarose 4 V/cm).

### EXAMPLE 3.

### Southern-hybridization

30 Transfer of the DNA to the hybridization membrane

The *Streptomyces* DNA preparations isolated above were digested with BamHI endonuclease (Boehringer Mannheim or New England Biolabs) according to the instructions of the manufacturer, and the digests were fractionated by agarose gel electrophoresis (Hopwood et al. 1985a, p. 137, TAE buffer, 0.8% agarose, 0,5 V/cm, running time 16 hours).



The fractionated DNA was transferred from the gel to Hybond N membrane (Amersham) using the VacuGen apparatus (LKB 2016, Pharmacia LKB Biotechnology) according to the instructions of the manufacturer (Preliminary Instruction Manual, LKB 2016 VacuGene Vacuum Blotting System, n:o 90 02 5378, Pharmacia LKB Biotechnology AB, Bromma, Sweden) with the modifications that depurination was performed for 10 min (in the instructions 4 min), denaturation for 15 min (3 min), neutralization for 15 min (3 min) and transferring for 1 h (20-60 min). The membrane, onto which the DNA had been transferred, was washed with 2 \* SSC, dried in room temperature and exposed to UV for 2 minutes by LKB 2011 Macro Vue Transilluminator (Pharmacia LKB Biotechnology) in order to fix the DNA.

15

#### Hybridization

The membranes to be hybridized were enclosed in a plastic bag (Hybaid) by heat-sealing. About 50 ml of a prehybridization solution was prepared:

20

- 1 % Na-dodecyl sulphate
- 1 M NaCl
- 5 \* Denhardt's solution

25

The hybridization bag was filled with such an amount of the prehybridization solution that air bubbles could be easily removed therefrom, and the prehybridization solution was poured from the bag into a container with a scale. Such an amount of a carrier DNA (DNA from calf thymus, Boehringer Mannheim 104 167) was denatured that the concentration in the prehybridization solution became 100 µg/ml, by heating in a boiling water bath for 10 min and by cooling in an ice bath for 5 min. The denatured carrier DNA was added into the prehybridization solution, which was returned to the hybridization bag, the air bubbles were removed as carefully as possible and pre-

30  
35

17

hybridization was performed at 65 °C in a shaking water bath at least for 6 h.

5 The labelled probe was added into an equal amount of the carrier DNA as above, and denatured similarly. The pre-hybridization mixture was removed from the bag and the denatured probe as well as the carrier were added there-  
10 to. The mixture was returned to the bag, the air bubbles were removed and hybridization was performed overnight in similar conditions as the prehybridization.

#### Washing and autoradiography

15 The hybridization mixture was removed from the bag and in its place 100 ml of washing solution was introduced:

- 1 % Na-dodecyl sulphate
- 2 \* SSC

20 The mixture was stirred, the washing solution was poured away, and the washing was repeated. Subsequently 300 ml of the washing solution was taken into the bag and it was shaken for 30 min in a 65 °C water bath. The washing solution was poured away and the washing was repeated.  
25 The membranes were spread on glass plates and covered by plastic film. The autoradiography was performed by placing one on top of the other the membrane protected by the plastic film, the autoradiography film (Hyperfilm-MP, Amersham) and the amplifying plate (Cronex Quanta Fast  
30 Detail, Dupont) and by exposing 1-2 days at -80 °C.

#### EXAMPLE 4.                      Preparation of the gene bank from *S. galilaeus* DNA

35 A gene bank was prepared into the  $\lambda$ -vector EMBL3 from the *S. galilaeus* DNA prepared as described above. The DNA was

partly digested with *Sau3A*-endonuclease (Maniatis et al., p. 282-285) and fractionated by sucrose gradient centrifuging according to the same instructions. The ca 20 kb DNA fraction was ligated with the vector (EMBL3 *Bam*HI Arms Cloning System, Promega Biotech.) according to the instructions of the manufacturer, and was packed to  $\lambda$ -particles by using the Packagene-reagent kit of the same manufacturer according to the instructions of the manufacturer. The *Escherichia coli* strain GM2163 (*E. coli* Genetic Stock Center, Department of Biology 255 OML, Yale University, New Haven, USA) was used as the host. The host cells were prepared for the infection according to the instructions of Maniatis et al. (p. 63). The host cells were infected with the obtained packing mixture and spread onto the plates according to the instructions of Promega.

EXAMPLE 5.                      Isolation of the acm sequence from the gene bank

From 150 mm plates, on which infected host cells were present so that plaques formed by the  $\lambda$ -phage amounted to about 10000 per plate, the phage DNA was immobilized to Colony/Plaque Screen (New England Nuclear) membranes according to the instructions of the manufacturer, so that two membranes were prepared from each plate. The membranes so obtained were hybridized and autoradiographed by using the act10.8 probe, as described above. Plaques giving a positive signal in autoradiography from both of the plates, were collected and the phages were eluted from them (Maniatis et al., p. 64).

In order to obtain pure positive phage populations a dilution was prepared from each of the candidates, which gave about 300 plaques/plate by infecting an *E. coli* host strain LE392 (Promega), the phage DNA was immobilized on

the membranes as described above, and hybridized as above. One clearly separate plaque for each candidate was collected, wherefrom the phages were eluted, and the host strain LE392 was infected by using such a dilution that so called confluent lysis was obtained on the plates. Phage base solutions were prepared from these plates, the titers of which, i.e. the concentration of the phages were determined (typically  $10^{10}$ /ml).

From each of the clones so obtained  $\lambda$ -phage was prepared in half a litre scale by infecting a NM538 host (Promega) according to the instructions described by Maniatis et al., p. 77-78.  $\lambda$ -phage DNA was prepared from the lysate according to the method of Kaslow (1986) with the modification that the phage DNA was precipitated with isopropanol rather than with polyethylene glycol (Hopwood et al. 1985a, p. 124).

#### EXAMPLE 6.

#### Preparation of the acm probe

The  $\lambda$ -DNA of the clones obtained was digested with BamHI endonuclease and it was fractionated by agarose gel electrophoresis, transferred onto a membrane, and hybridized using the act10-8 probe as described above for the chromosomal DNA. One of the clones, designated  $\lambda$ -acm5, gave a ca 3 kb BamHI fragment, which gave a distinct hybridization signal; the fragment hybridizing with the other positive clones did not separate from the vector with BamHI endonuclease.

$\lambda$ -acm5-DNA was digested with BamHI endonuclease and the digest was fractionated by preparative agarose gel electrophoresis. The 3 kb BamHI fragment was isolated and purified as described above, and it was ligated into the plasmid vector pBR322 (Bolivar et al., 1977 and Sutclif-

fe, 1978) opened with *Bam*HI endonuclease (Maniatis et al., p. 391). The plasmid is commonly available, a.o. in the *E. coli* strain ATCC 37017 of American Type Culture Collection.

5

Competent *E. coli* HB101 cells were transformed with the ligation mixture (Hopwood et al. 1985a, p. 120) and among the obtained transformants a clone containing the 3 kb *Bam*HI fragment was searched by preparing plasmid DNA from the clones in a small scale (Hopwood et al. 1985a, p. 85), by digesting the samples with *Bam*HI endonuclease and by identifying the clones having the insert by agarose gel electrophoresis. The plasmid carrying the insert was designated *pacm*5, and it was prepared in a large scale, as described above for the plasmid pIJ2345. The probe, abbreviated *acm*, is the 3 kb *Bam*HI fragment contained in the plasmid *pacm*5, which fragment was isolated as described for the *act*I0.8 probe from pIJ2345.

20

EXAMPLE 7.Preparation of the gene bank from *S. purpurascens* DNA

The gene bank was prepared from *S. purpurascens* DNA as described above for *S. galilaeus*, however so that the vector used was  $\lambda$ -EMBL4, and as the vector preparation " $\lambda$ -EMBL4 *Bam*HI Arms", RPN.1254X, Amersham International plc was used.

30

EXAMPLE 8.Screening of the gene bank of *S. purpurascens* and mapping of the clones

The clones giving a positive hybridization signal were screened from the *S. purpurascens* gene bank by the same procedure as described above for the *S. galilaeus* gene bank by using the *acm* probe, the clones were purified,

and phage base solutions were prepared thereof. From the clones  $\lambda$ -DNA was prepared as described above, and the clones were mapped for some restriction endonuclease recognition sites (Maniatis et al., p. 374-378). The so  
5 obtained map from the clones, which were designated by the abbreviation *rdm* and a serial number, is given in the Figure 3.

EXAMPLE 9.                      Transfer of the *S. purpurascens* DNA  
10                                      sequences to *S. galilaeus* and disco-  
   very of the producer of hybrid anti-  
   biotics

The transfer of the inserts of the clones obtained into  
15 *Streptomyces galilaeus* was initiated for detection of possible new products. As *S. galilaeus* evidently has a fairly strong restriction system, it was not possible to transform the ligation mixtures directly to *S. galilaeus*, but the clones had to be transferred first to an easily  
20 transformable *Streptomyces* host, as which *S. lividans* TK24 (Hopwood et al. 1985a, p. 266 and Kieser et al. 1982) was used, which strain was obtained from professor D.A. Hopwood, John Innes Institute (Norwich, UK).

25 The recombinant phage  $\lambda$ -*rdm6* was digested with *EcoRI* (Boehringer Mannheim) according to the instructions of the manufacturer. An insert of ca 12 kb was run separate from the arms on 0.5% SeaPlaque LGT agarose gel (FMC Corporation) at 8 V voltage overnight. The insert DNA was  
30 recovered from the gel using a GeneClean reagent kit (Biol01) according to the instructions of the manufacturer.

35 The *Streptomyces* plasmid pIJ486 (Ward et al., 1986; the plasmid was obtained from prof. Hopwood, John Innes Institute) was linearized with *EcoRI* as the recombinant

phage and treated with alkaline phosphatase (Calf Intestinal Alkaline Phosphatase, CIAP, Boehringer Mannheim 713023) as follows: 10 µg of linear vector DNA and 0.5 U of CIAP were incubated in a +37 °C water bath in a volume of 100 µl (50 mM Tris-HCl, pH 8 and 0.1 mM EDTA) 0.5 h. CIAP was inactivated by heating at 65 °C for ca 0.5 h and by extracting subsequently two times with a neutral phenol-chloroform (1:1) mixture. The plasmid was precipitated with 2-propanol, washed with ca 70% ethanol and dissolved in TE buffer (about 1 µg/µl).

The plasmid pIJ486 and the insert-DNA were ligated with T4-DNA-ligase (Boehringer Mannheim) according to the instructions of the manufacturer in a volume of 20 µl.

The ligation mixture was transformed into *S. lividans* TK24 protoplasts stored in -20 °C. The preparation and the transformation of the protoplasts was performed according to the method described by Hopwood et al. (1985a) (pages 12-14 and 108-109). After 1 day, thio-streptone (tsr) was added onto the plates as a water suspension, 0.5 mg/plate. The regenerated protoplasts were collected with tooth picks after ca 6 days to ISP4 plates (Difco), on which 50 µg/ml of thiostreptone had been added. Some of the culture was transferred with a loop from the ISP4 plates to Falcon tubes, which contained 3 ml of TSB medium (Oxoid Tryptone Soya Broth 30 g/l) and 5 µg/ml of thiostreptone, in order to maintain the selection pressure. After 3 days, plasmids were isolated from 42 cultures according to the method described by Kieser (1984). 8 of the isolated plasmids carried the insert. One of these (pH2008) was grown in 500 ml of TSB medium, and the plasmid was isolated therefrom according to the method of Kieser. The map of the plasmid pH2008 is given in Figure 4.

The modifications to the above mentioned transformation method used for *S. lividans*, which are needed for the transformation of *Streptomyces galilaeus*, have been described earlier (Ylihonko, K. Thesis for M.Sc., University of Turku, 1986). To prepare the protoplasts, SGYEME medium was used instead of YEME medium (see above), wherein 0.8 % of glycine had been added. 1-2 µg of the plasmid preparation was transformed into the *S. galilaeus* strain ATCC 31615. One transformant was obtained.

The strain ATCC 31615/pH2008 was grown in E1 growth medium, whereto 5 µg/ml of thiostreptone had been added and after 7 days the products were extracted from the culture with a toluene-methanol (1:1) mixture. As a control, the products of the strain ATCC 31615 were isolated correspondingly. About 1 ml of the culture was used for the isolation of the products, wherefrom the cells were separated from the medium by centrifuging. Only the cells were used for the extraction, because only a small part of the products were left in the supernatant. The products were separated from the toluene phase of the extract by thin layer chromatography, using as the eluant the mixture toluene - ethyl acetate - methanol - formic acid (50:50:15:10). It was found that the strain ATCC 31615/pH2008 had formed anthracyclines, which the host strain ATCC 31615 does not produce naturally.

It was established that the characteristics were derived from the plasmid by retransforming the recombinant plasmid pH2008 to ATCC 31615, whereby it was found that the new compounds were formed in this strain as well. Growing the recombinant strain in the production medium E1 without the selection pressure caused by thiostreptone led to the disappearance of the production of the new anthracyclines.



The hybrid products were hydrolyzed by heating them for 0.5-1 hours in 0.1M HCl solution. The aglycones were separated on a TLC plate treated with oxalic acid, and they were compared to the aglycones produced by the strain ATCC 31615. Chloroform-acetone (10:1) was used as the eluent. The Rf values given in the Table I were obtained for the products:

Rf value	ATCC 31615	ATCC 31615/pH2008
0.11	-	+
0.15	-	+
0.19	-	+
0.21	+	+
0.30	-	+
0.36	-	+
0.42	-	+
0.46	+	+
0.50	-	+
0.53	-	+
0.56	+	+
0.61	-	+

- = The strain does not contain the product  
+ = The strain produces a product corresponding to the Rf value

Table I.

EXAMPLE 10.                      Production of hybrid antibiotics and purification of the aglycones

**Fermenting**

5

*S. galilaeus* ATCC 31615/pH2008 was inoculated from the ISP4 + tsr plate to a shaking flask, which contained 60 ml of the medium E1, whereto 5 µg/ml of thiostreptone had

been added, and it was grown for 4 days in a shaker at 300 rpm at a temperature of 30 °C. A 7 l fermentor was prepared, which contained 5,5 l of the medium E1, 5 ml of anti-foam substance (Polypropylenglycol P 2000, Fluka) and 5 µg/ml of thiostreptone. The fermentor was inoculated with the above mentioned preculture and fermented for 5 days at a temperature of 30 °C by stirring at 350 rpm and using an air supply of 6 l/min.

## 10 Extraction

A 10 l reaction flask was prepared for the extraction by adding thereto:

	Celatom <sup>(R)</sup>	400	g
15	Na <sub>2</sub> HPO <sub>4</sub> · 2H <sub>2</sub> O	47	g
	citric acid	24.4	g
	water	500	g
	methylethylketone (MEK)	3	l

20 The culture broth was transferred from the fermentor by vacuum to the extraction container and stirred for 45 min. The mixture was filtered in a Büchner funnel and the filter cake was washed with 300 ml of MEK. In the filtrate, 500 g of NaCl was dissolved and was allowed to separate overnight in a separatory funnel.

The lower phase (water phase) was removed. The upper phase was run into a container, where to 400 g of Na<sub>2</sub>SO<sub>4</sub> had been weighed. It was stirred for 10 min and filtered in a Büchner funnel. The filter cake was washed with 200 ml of MEK.

## Separation of the glycosides

35 The raw MEK extract so obtained was evaporated by rotary evaporator to nearly dryness. 200 ml of toluene was added and evaporated to dryness. The mixture was filled with

toluene to 150 ml. 150 ml of isopropanol, 150 ml of 0.1 M HCl and 75 ml of hexane were added.

The mixture was transferred into a separatory funnel,  
5 stirred and the layers were allowed to separate. The lower layer (water phase) was taken into another separatory funnel, and 40 ml of dichloromethane was added thereto. It was stirred and the phases were allowed to separate. The lower phase (dichloromethane) was run into  
10 a container, which contained 30 ml of 1M phosphate buffer, pH 7.0. To the water phase left in the separatory funnel 10 ml of dichloromethane was added, extracted, and the MeCl<sub>2</sub> phase was pooled with the MeCl<sub>2</sub> phase under the above mentioned phosphate buffer. To the toluene phase of  
15 the first extraction 75 ml of isopropanol and 150 ml of 0.1 M HCl were added.

The extraction into methylene chloride was repeated as above and the methylene chloride phases were combined.  
20 The methylene chloride solution was evaporated in a rotary evaporator to dryness. About 10 ml of methanol was added and reevaporated to dryness. The evaporation residue was dissolved in 200 ml of toluene.

#### 25 Hydrolysis of the glycosides to aglycones

To the above obtained toluene solution 200 ml of 0.1M HCl was added, stirred and the water phase was separated. The water phase was incubated for 2 hours in a 85 °C water  
30 bath. The solution was cooled and it was extracted three times with 100 ml of toluene. The combined toluene phase was then extracted with 150 ml of 0.1M Na-phosphate buffer, pH 7.0. The toluene phase was evaporated to dryness in a rotary evaporator, 20 ml of methanol was  
35 added, the mixture was reevaporated to dryness, and it was dissolved in 10 ml of toluene.

### Chromatographic separation of the aglycones

The aglycones were separated chromatographically in two steps. The first chromatographic run was performed in an  
5 oxalic acid - silica gel column as follows:

The oxalic acid - silica gel was prepared by mixing 100 g of Kieselgel 60 (Merck) with 200 ml of 0.25M oxalic acid, by removing most of the oxalic acid solution in a Büchner  
10 funnel and by drying the silica gel overnight in a 110 °C hot cupboard. The oxalic acid - silica gel so obtained was slurried in toluene and packed into a 4 cm diameter chromatographic column.

15 The aglycon mixture in toluene obtained above was applied to the column and eluted with a solution, which contained 10 % acetone in toluene. About 15 ml fractions were collected from the eluate and the fractions were analyzed by thin layer chromatography as described above. Fig. 5  
20 shows a photograph of the thin layer so obtained, on the basis of which the eluate was divided into four fractions: I, II, III and IV.

The fractions I and II seemed to contain two main components, wherefore they were rechromatographed using  
25 oxalic acid - silica gel, but the column used had a diameter of 1.5 cm and length of ca 50 cm. For the elution, 5 % acetone in methylene chloride was used. Thus the main components of the fractions I and II could be  
30 purified, which were designated IA, IB, IIA and IIB.

The aglycones obtained were chrystallized from methanol.

### Structure determination of the aglycones

35

The obtained aglycone fractions were characterized by thin layer chromatography, mass spectrometry and proton

NMR. IA was found to be aklavinone, i.e. the aglycone produced by the host strain ATCC 31615. IB is 6-rhodomyacinone, which is the aglycone produced by the gene donor strain *S. purpurascens*, as well as IIB, which is  $\beta$ -rhodomycinone. IIA is 10-decarbomethoxy aklavinone, which has been described as the product of chemical demethylation and decarboxylation of aklavinone (Tanaka et al., 1980), but the inventors have not found any information in the literature, that any microbe would produce it. III was found to be the 7-epimer of IIA, and it has evidently been formed in the connection with the hydrolysis.

The structure given in Fig. 6 in connection with the proton-NMR-spectrum was obtained for the aglycon IV, which structure the inventors have not found in the literature.

The aglycones IIA and IV are thus products, which are neither produced naturally by the gene recipient strain nor the donor strain, i.e. they are hybrid antibiotics. Aglycone IV is also absolutely new.

EXAMPLE 11.                      Production of the hybrid antibiotics and purification of the glycosides

25    Fermenting, extracting and separation of the glycosides was performed as in the Example 10.

Purification of the glycosides IVA and IVB

30    2 ml of the above obtained glycoside extract was applied as a narrow band onto a thin layer chromatography plate (20 cm x 20 cm x 0.5 mm, Kieselgel 60, Merck). The plate was eluted with the eluant chloroform:methanol:acetic acid (20:5:1). The compounds IVA (yellow product, R<sub>f</sub> value 0.40) and IVB (yellow product, R<sub>f</sub> value 0.55) were  
35    scratched from the plate and extracted into methanol.

### Partial hydrolysis of the glycosides

About 200 ml of the glycoside extract was extracted by adding 200 ml of 0.05M HCl, by stirring and separating  
5 the water phase. The water phase was incubated for 30 min on a 55 °C water bath. After the incubation the solution was neutralized by adding 20 ml of 1M phosphate buffer, pH 7.

10 The solution was extracted three times with 100 ml of chloroform. The chloroform phase was evaporated to dryness and dissolved in 20 ml of dichloromethane.

### Purification of IVT

15 IVT was purified chromatographically in two steps from the partly hydrolyzed glycoside fraction obtained above. Both of the steps were performed in a silica gel column.

20 In the first step Kieselgel 60 was slurried into dichloromethane and packed into a column of 4 cm diameter. The glycoside mixture obtained above was applied to the column and eluted with the eluant dichloromethane:methanol:acetic acid (100:20:1). About 15 ml fractions were  
25 collected from the eluate and the fractions were analyzed by thin layer chromatography using the eluant chloroform:methanol:acetic acid (20:5:1). The fractions containing mainly IVT (yellow product, Rf 0.21) were pooled. Acetic acid was extracted from the pooled fractions into  
30 water by neutralizing the solution with 1M NaOH solution. The yellow dichloromethane phase was evaporated to dryness and dissolved in 2 ml of methanol, which was mixed with 20 ml of toluene.

35 The anthracycline in the toluene-methanol solution was applied in the second step onto a chromatography column prepared from Kieselgel 60 slurried in toluene. The

column was eluted with the eluant toluene:methanol (1:1) and ca 15 ml fractions were collected. On the basis of thin layer chromatography the fractions containing IVT were selected as above. The fractions were combined and  
5 evaporated to dryness.

#### Structure determination of the glycosides IVA, IVB and IVT

10 IVA, IVB and IVT were found to give aglycon IV when hydrolyzed with 1M HCl on a boiling water bath. IVA and IVB were found to give IVT when hydrolyzed with 0.05M HCl for 30 min on a 55 °C water bath. By this treatment IVT converted in small amounts to aglycone. On the basis of  
15 the mass and HNMR spectra (Figs 8 and 9) IVT proved to be a glycoside with one sugar, the sugar moiety of which is rhodnose.

#### Biotransformation of glycoside IVB to IVA

20

About 1 mg of IVB was dissolved in 1 ml of methanol.

60 ml of a ATCC 31615 culture, which had been grown for 2 days in E1 medium, was washed by centrifuging and sus-  
25 pending the cells into 60 ml of a physiological saline solution and transferred to a shaking flask. IVB dissolved in methanol was added to the flask and the flask was shaken for 6 h at 300 rpm at a temperature of 30 °C. After incubation, the products were extracted from the  
30 culture as in the Example 9. On the basis of thin layer chromatography, IVB was found to have converted to IVA.

It has been found that the strain ATCC 31615 under these conditions converts aclacinomycin B to A (Hoshino et al.,  
35 1983). The reaction has been found to occur also in compounds whose aglycone moiety differs from aklavinone, but the sugar moiety is the same as in aclacinomycin B.

On the basis of this reaction it can be established that the structure of IVA corresponds to that of aclacinomycin A and the structure of IVB that of aclacinomycin B. Also the results obtained from the hydrolysis support this structure determination.

5 EXAMPLE 12.

Biological activity of the glycosides  
IVA, IVB and IVT

10 The activities of the compounds were determined by a cytotoxicity test, in which the ability of the compounds to inhibit growth of the mouse leukemia cell line L1210 (ATCC CCL 219) *in vitro* (Matsuzawa et al., 1981) was measured. As the comparative substance aclacinomycin A  
15 was used. The following ED<sub>50</sub> values were obtained for the compounds:

	Compound	ED <sub>50</sub> /nmol/l
20	Aclacinomycin A	10
	IVA	75
	IVB	24
	IVT	22



DEPOSITED MICROORGANISMS

The following microorganism strains have been deposited according to the Budapest treaty to Deutsche Sammlung von  
5 Mikroorganismen und Zellkulturen GmbH (DSMZ). Mascheroder  
Weg 1 B, D-3300 Braunschweig, Germany.

	Microorganism	Deposition number	Deposition date
10	<i>Streptomyces galilaeus</i> ATCC 31615/pH2008	DSM 6403	March 4, 1991
15	<i>Escherichia coli</i> HB101/pacm5	DSM 6404	March 4, 1991

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## Claims

1. DNA sequence useful in obtaining hybrid antibiotics of the anthracycline group, characterized in that it has  
5 been isolated from a *Streptomyces purpurascens* bacterium producing anthracyclines and as transferred into a *Streptomyces galilaeus* host producing naturally glycosides of aklavinone, causes the production of such anthracyclines which are not produced naturally by this strain.

10 2. DNA sequence as claimed in claim 1, characterized in that it has been isolated from *Streptomyces purpurascens* strain ATCC 25489.

15 3. DNA sequence as claimed in claim 2, characterized in that it is the ca 12000 bp sequence between the two *EcoRI*-restriction endonuclease recognition sites of the plasmid pH2008, or a functional fragment thereof.

20 4. Recombinant-DNA-construction useful in obtaining hybrid antibiotics of anthracycline group, characterized in that the DNA-sequence according to the claims 1 to 3 is inserted into a vector which is amplified in micro-organisms of the genus *Streptomyces*.

25 5. Recombinant-DNA-construction according to the claim 4, characterized in that it is the plasmid pH2008, the restriction map of which is given in Fig. 4, and which can be isolated from *Streptomyces galilaeus* strain DSM  
30 6403.

6. *Streptomyces* strain producing hybrid antibiotics of the anthracycline group, characterized in that the recombinant-DNA-construction according to the claim 4 or 5  
35 has been introduced into a *Streptomyces galilaeus* strain naturally producing glycosides of aklavinone.

7. *Streptomyces* strain according to the claim 6, characterized in that the *Streptomyces galilaeus* strain used as the host and producing glycosides of aklavinone is ATCC 31615 or ATCC 31133.

5

8. *Streptomyces* strain according to the claim 7, characterized in that the vector is pIJ486.

9. *Streptomyces* strain according to any one of the claims 6 to 8, characterized in that the recombinant-DNA-construction is the plasmid pH2008.

10

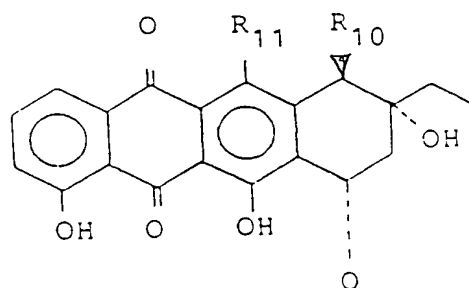
10. *Streptomyces galilaeus* DSM 6403.

11. Process for producing hybrid antibiotics of the anthracycline group, characterized in that a *Streptomyces* strain according to any of the claims 6 to 9 is grown under conditions where the host strain naturally produces antibiotics, the produced antibiotics are separated from the growth medium and the hybrid antibiotics obtained are purified.

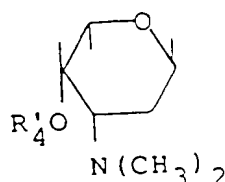
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12. Process according to the claim 11, characterized in that the produced compounds correspond to their structure to the following general formula:

25



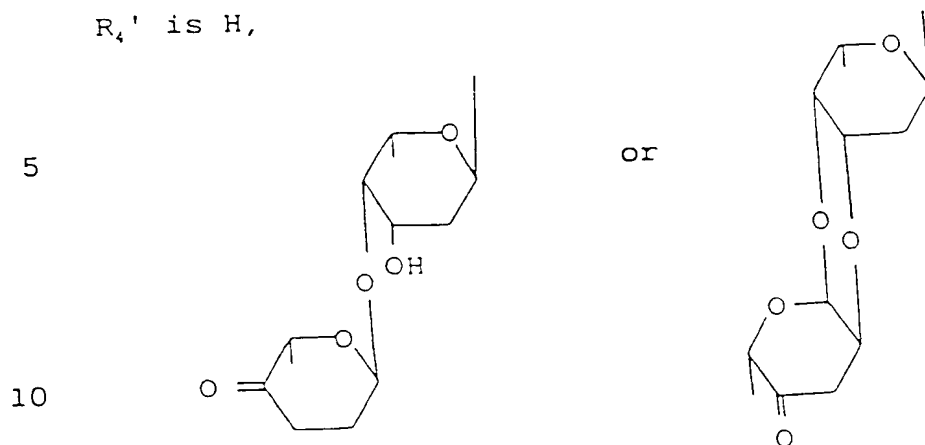
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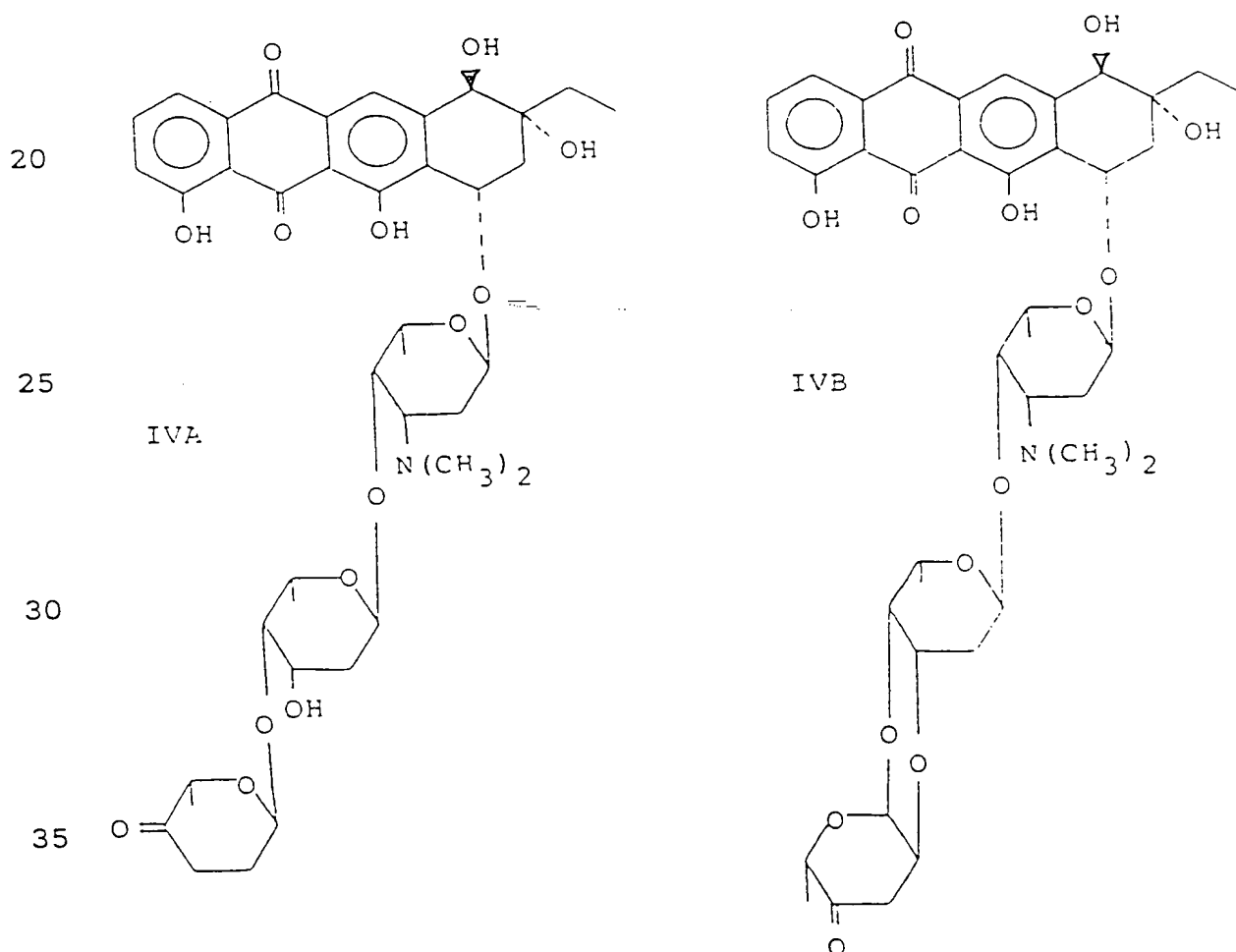
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wherein  $R_{10}$  is H or OH,  $R_{11}$  is H or OH and  $R_4'$  is H,



13. Process according to the claim 12, characterized in that the produced hybrid antibiotic is IVA, IVB or IVT; the structural formulas of which are

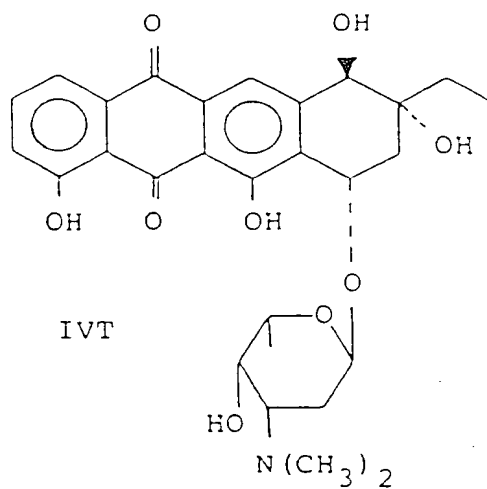
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14. Compounds IVA, IVB and IVT, the structural formulas of which are

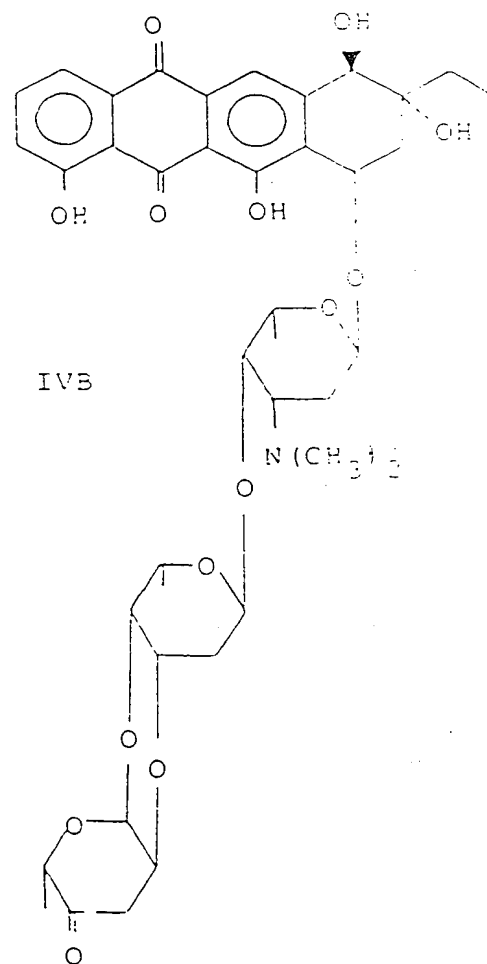
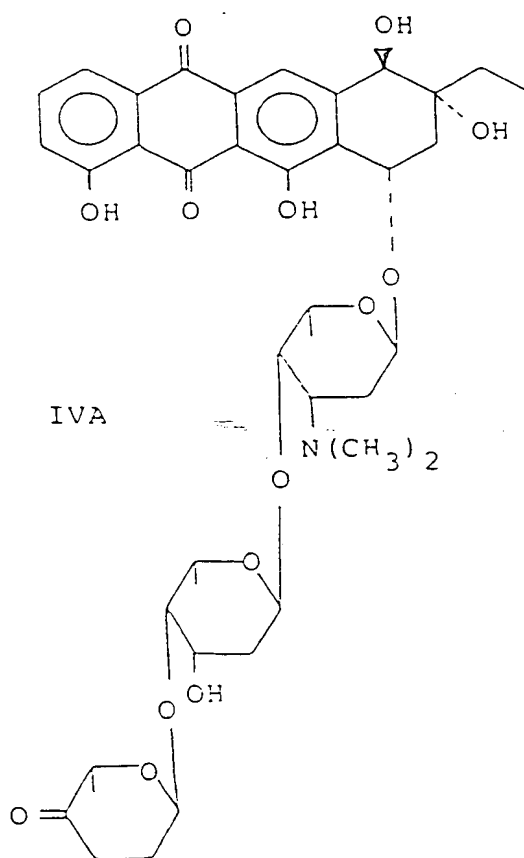
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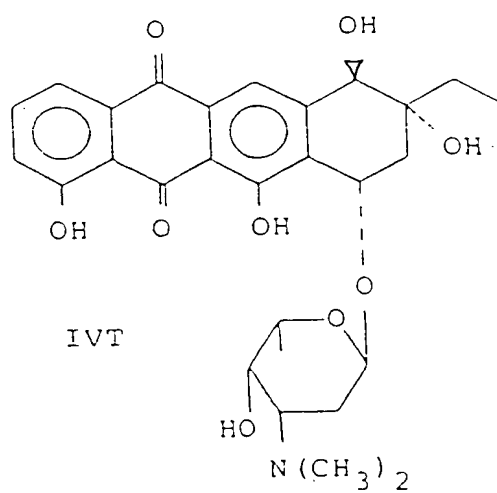
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## AMENDED CLAIMS

[received by the International Bureau on 25 August 1992 (25.08.92); original claims 1 and 4 amended; other claims unchanged (5 pages)]

1. DNA sequence useful in obtaining hybrid antibiotics of the anthracycline group, characterized in that it has  
5 been isolated from a *Streptomyces purpurascens* bacterium producing anthracyclines, by
  - a) isolating from *S. galilaeus* DNA a fragment hybridizing with act10.8 probe, and
  - b) using the fragment so obtained in the hybridization  
10 of *S. purpurascens*,  
and as transferred into a *Streptomyces galilaeus* host producing naturally glycosides of aklavinone, causes the production of such anthracyclines which are not produced naturally by this strain.  
15
2. DNA sequence as claimed in claim 1, characterized in that it has been isolated from *Streptomyces purpurascens* strain ATCC 25489.
- 20 3. DNA sequence as claimed in claim 2, characterized in that it is the ca 12000 bp sequence between the two *EcoRI*-restriction endonuclease recognition sites of the plasmid pH2008, or a functional fragment thereof.
- 25 4. Recombinant-DNA-construction useful in obtaining hybrid antibiotics of anthracycline group, characterized in that the DNA-sequence according to the claims 1 to 3 is inserted into a vector which is amplified in micro-organisms of the genus *Streptomyces*.  
30
5. Recombinant-DNA-construction according to the claim 4, characterized in that it is the plasmid pH2008, the restriction map of which is given in Fig. 4, and which can be isolated from *Streptomyces galilaeus* strain DSM  
35 6403.

6. *Streptomyces* strain producing hybrid antibiotics of the anthracycline group, characterized in that the recombinant-DNA-construction according to the claim 4 or 5  
5 has been introduced into a *Streptomyces galilaeus* strain naturally producing glycosides of aklavinone.

7. *Streptomyces* strain according to the claim 6, characterized in that the *Streptomyces galilaeus* strain used as  
10 the host and producing glycosides of aklavinone is ATCC 31615 or ATCC 31133.

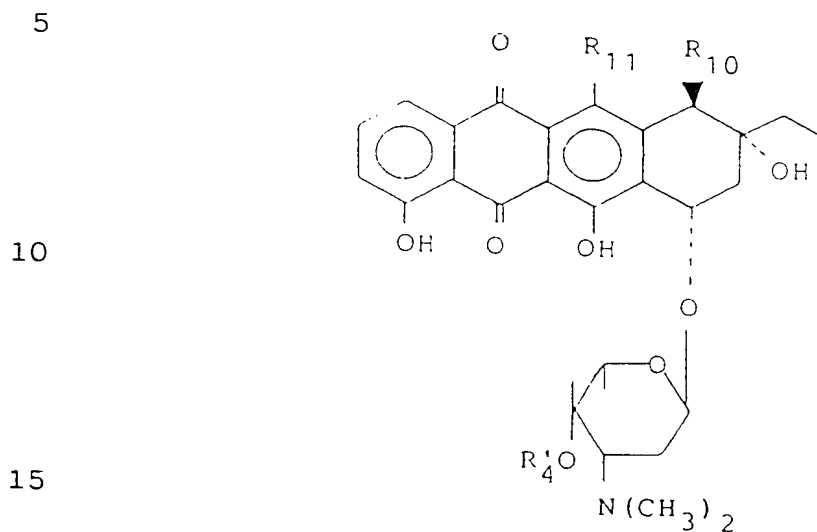
8. *Streptomyces* strain according to the claim 7, characterized in that the vector is pIJ486.  
15

9. *Streptomyces* strain according to any one of the claims 6 to 8, characterized in that the recombinant-DNA-construction is the plasmid pH2008.

20 10. *Streptomyces galilaeus* DSM 6403.

11. Process for producing hybrid antibiotics of the anthracycline group, characterized in that a *Streptomyces* strain according to any of the claims 6 to 9 is grown  
25 under conditions where the host strain naturally produces antibiotics, the produced antibiotics are separated from the growth medium and the hybrid antibiotics obtained are purified.

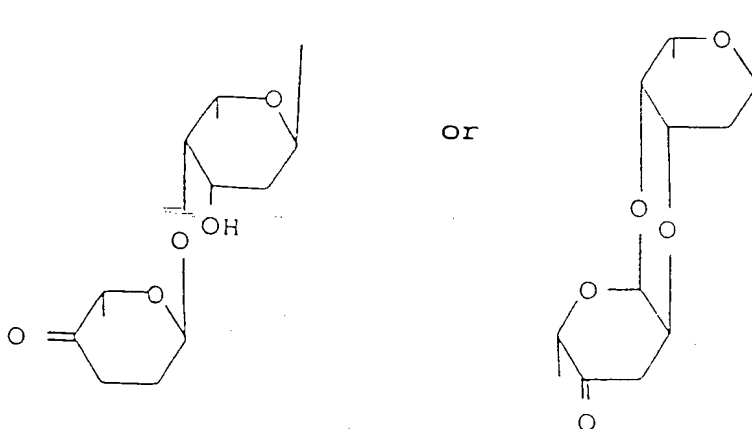
12. Process according to the claim 11, characterized in that the produced compounds correspond to their structure to the following general formula:



wherein  $R_{10}$  is H or OH,  $R_{11}$  is H or OH and  $R_4'$  is H,

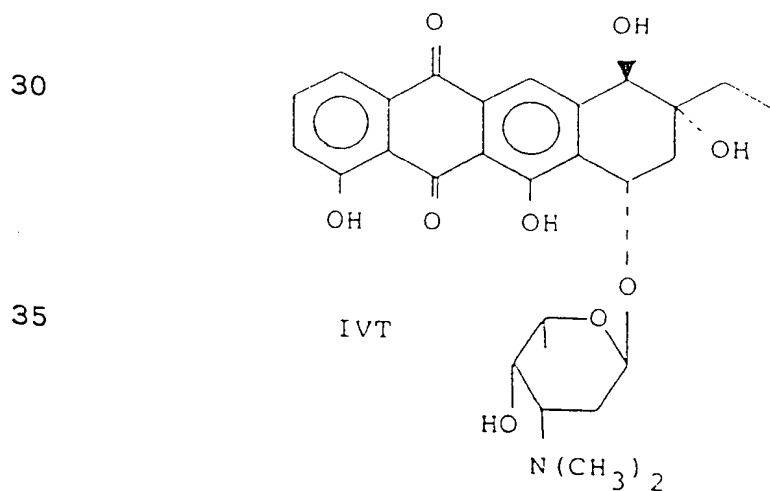
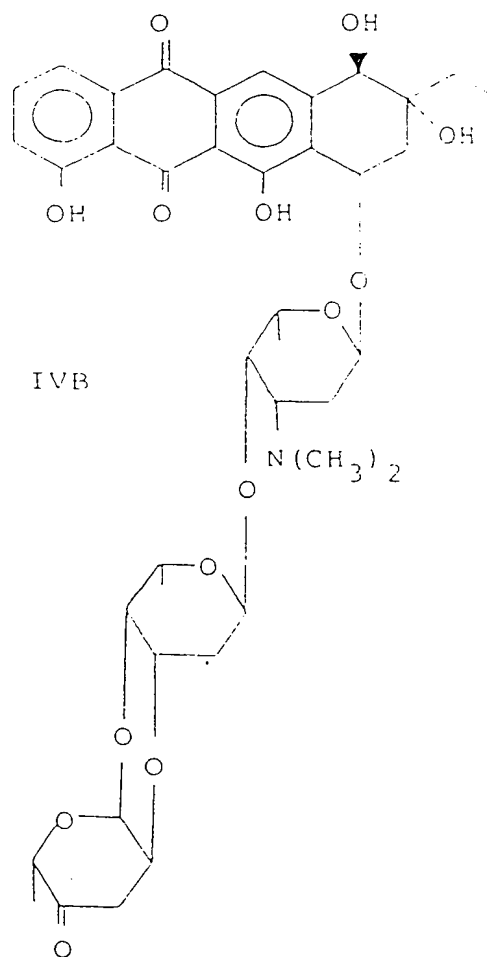
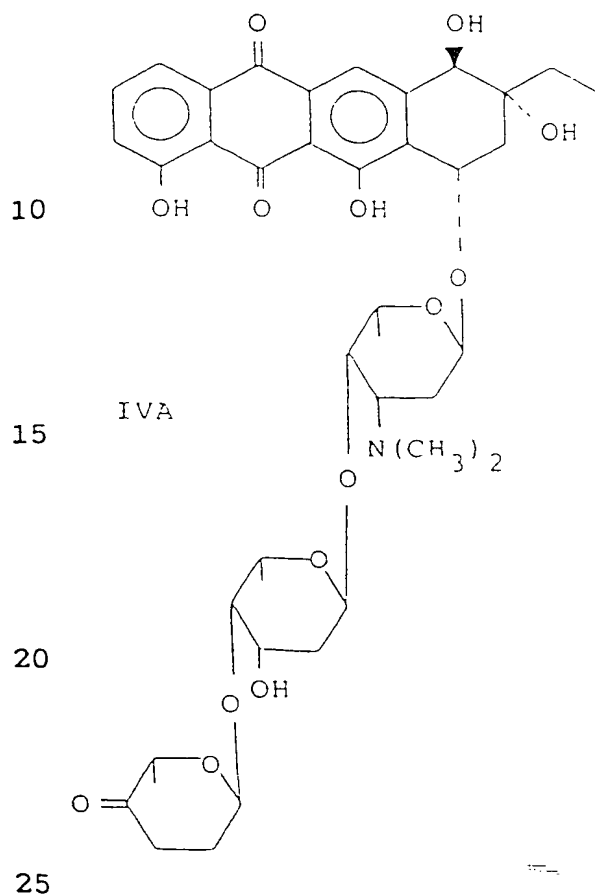
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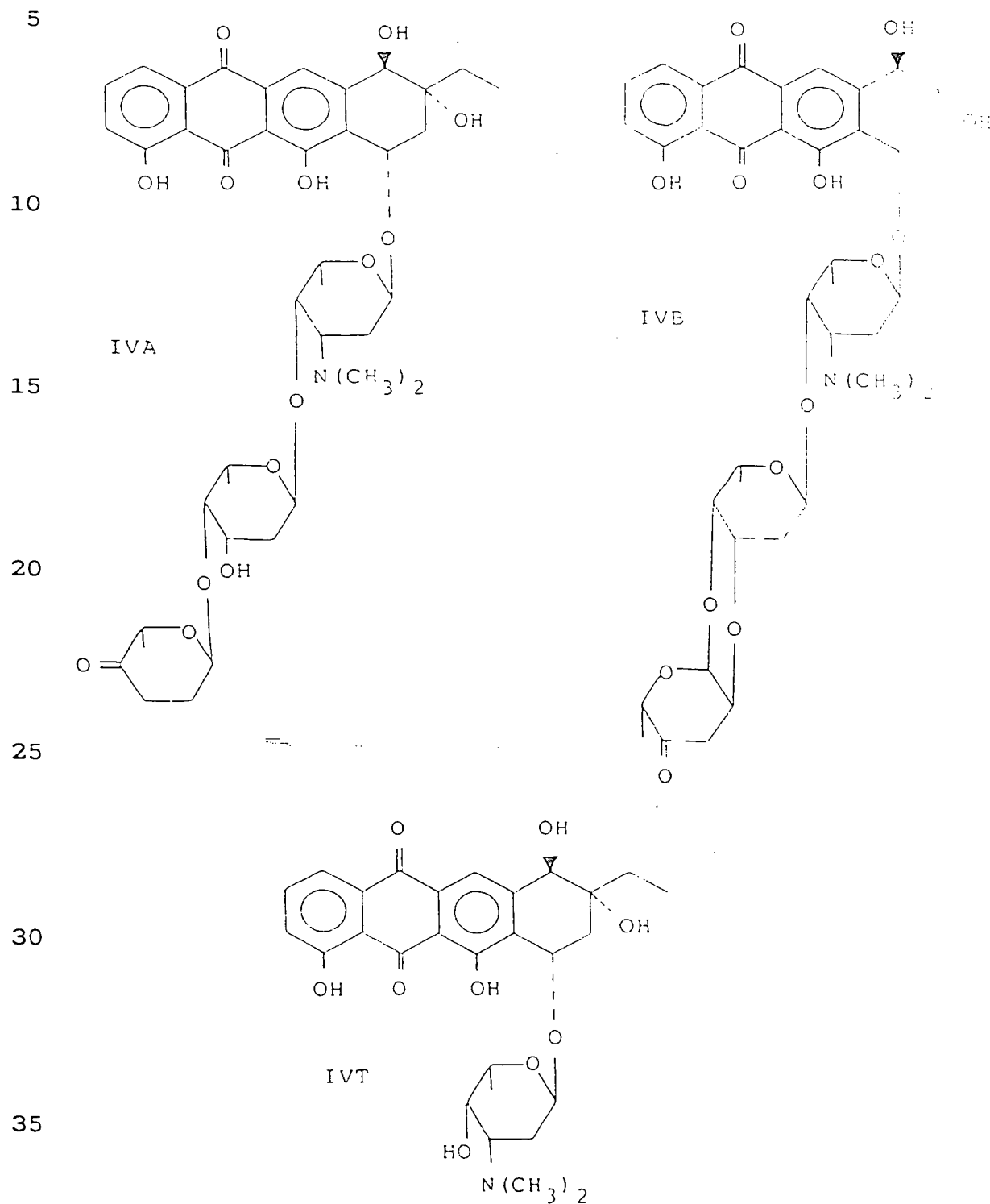


13. Process according to the claim 12, characterized in that the produced hybrid antibiotic is IVA, IVB or IVT, the structural formulas of which are

5



14. Compounds IVA, IVB and IVT, the structural formulas of which are



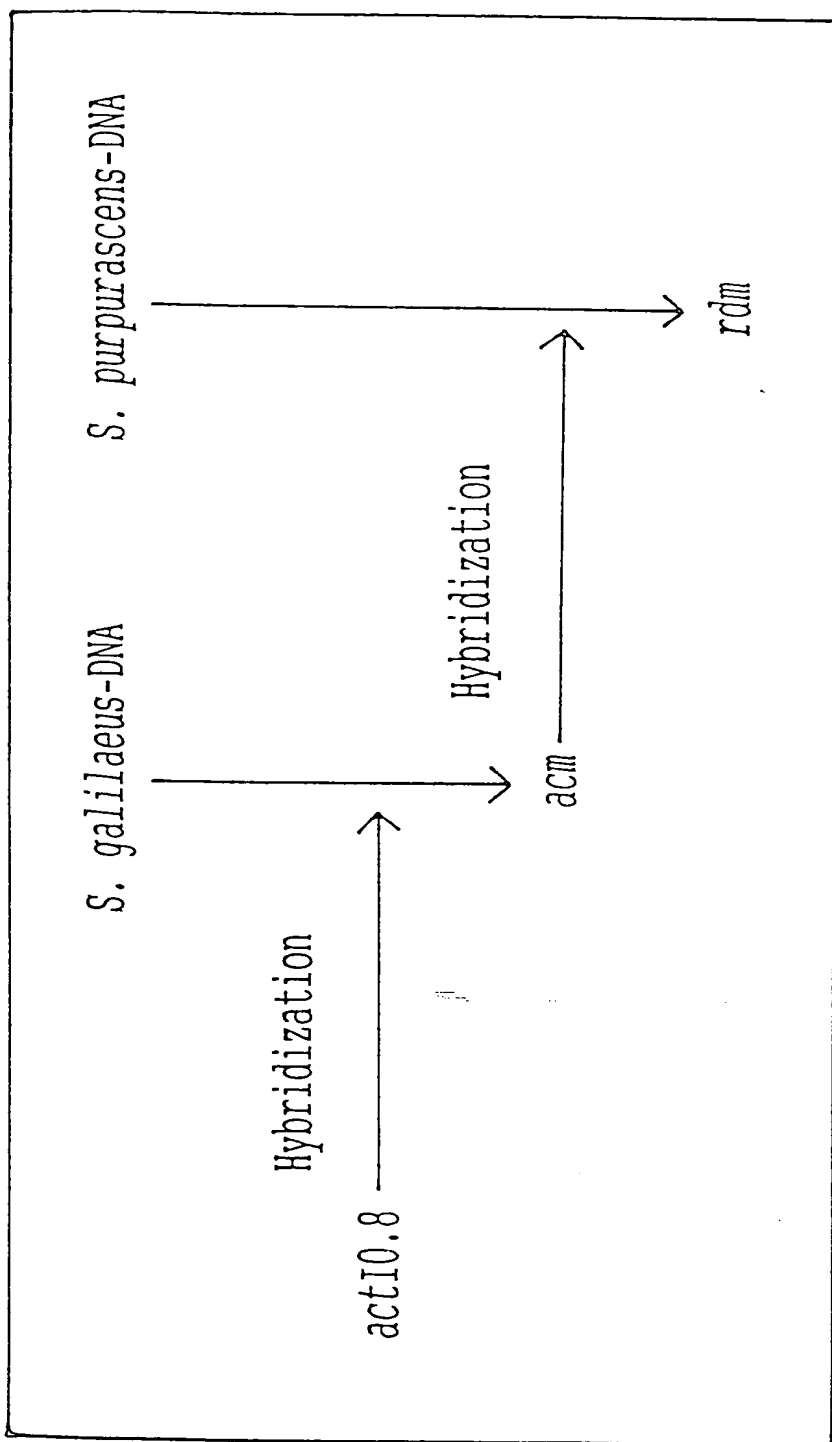


FIG. 1

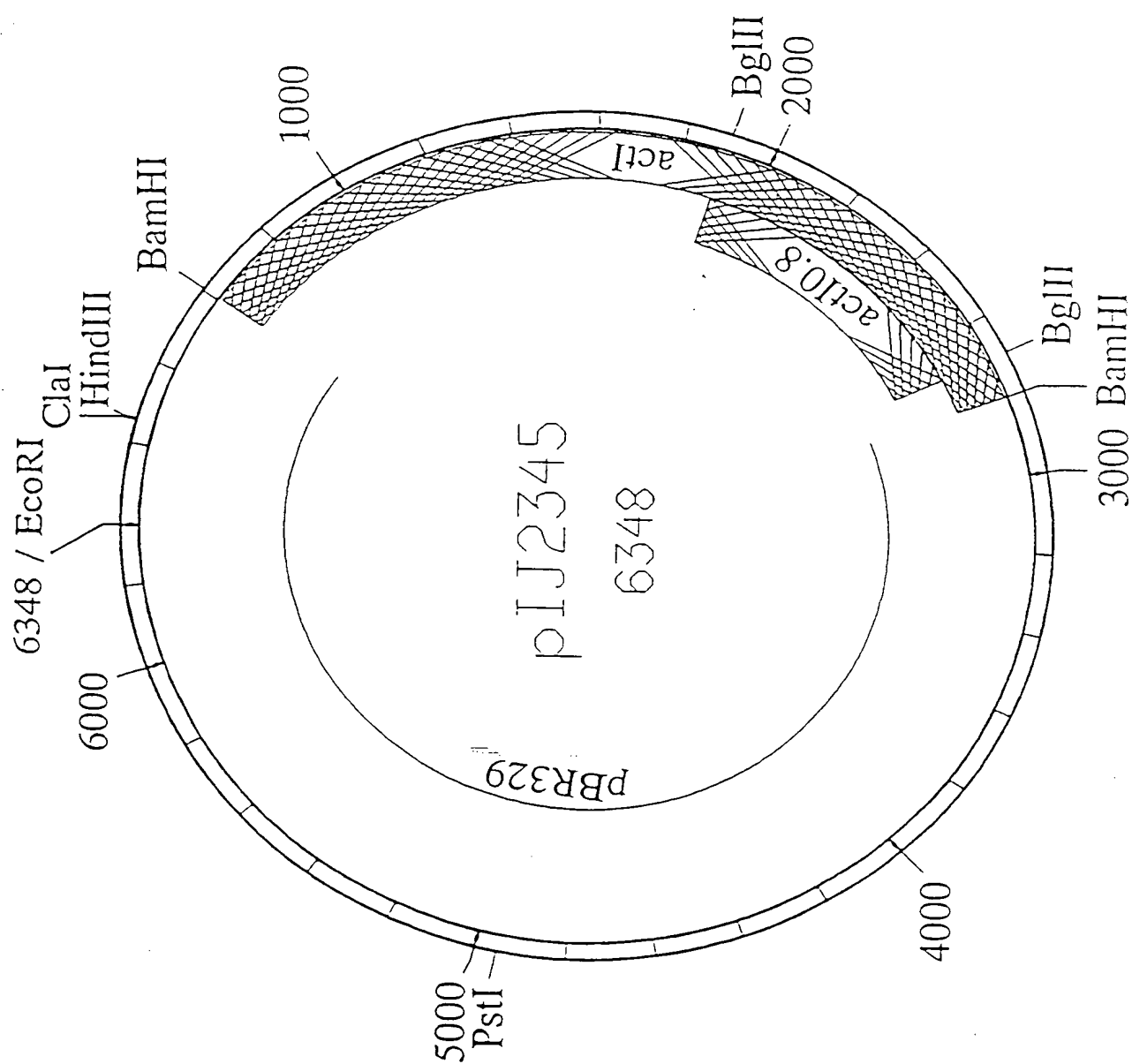


FIG. 2

3/9

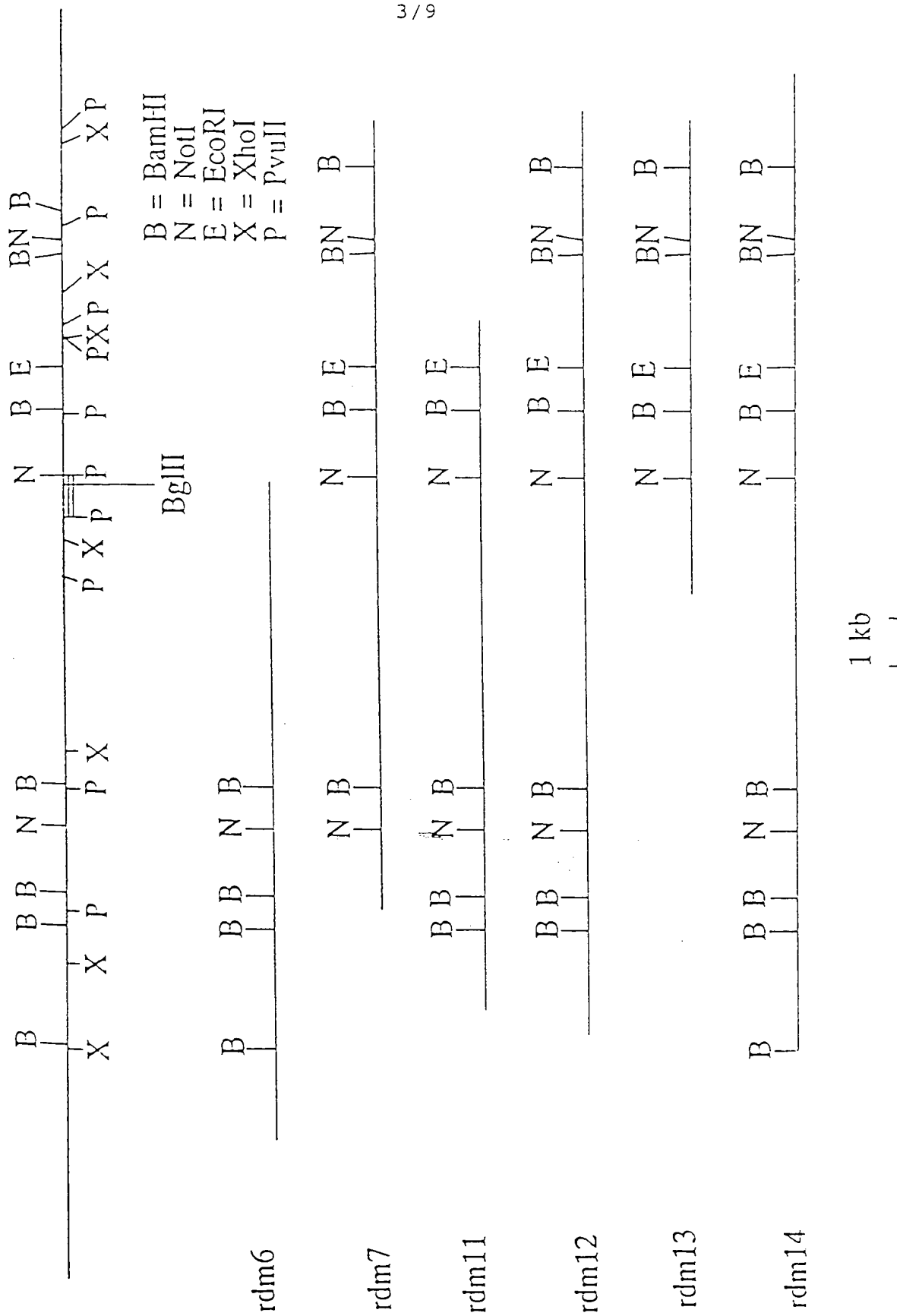


FIG. 3



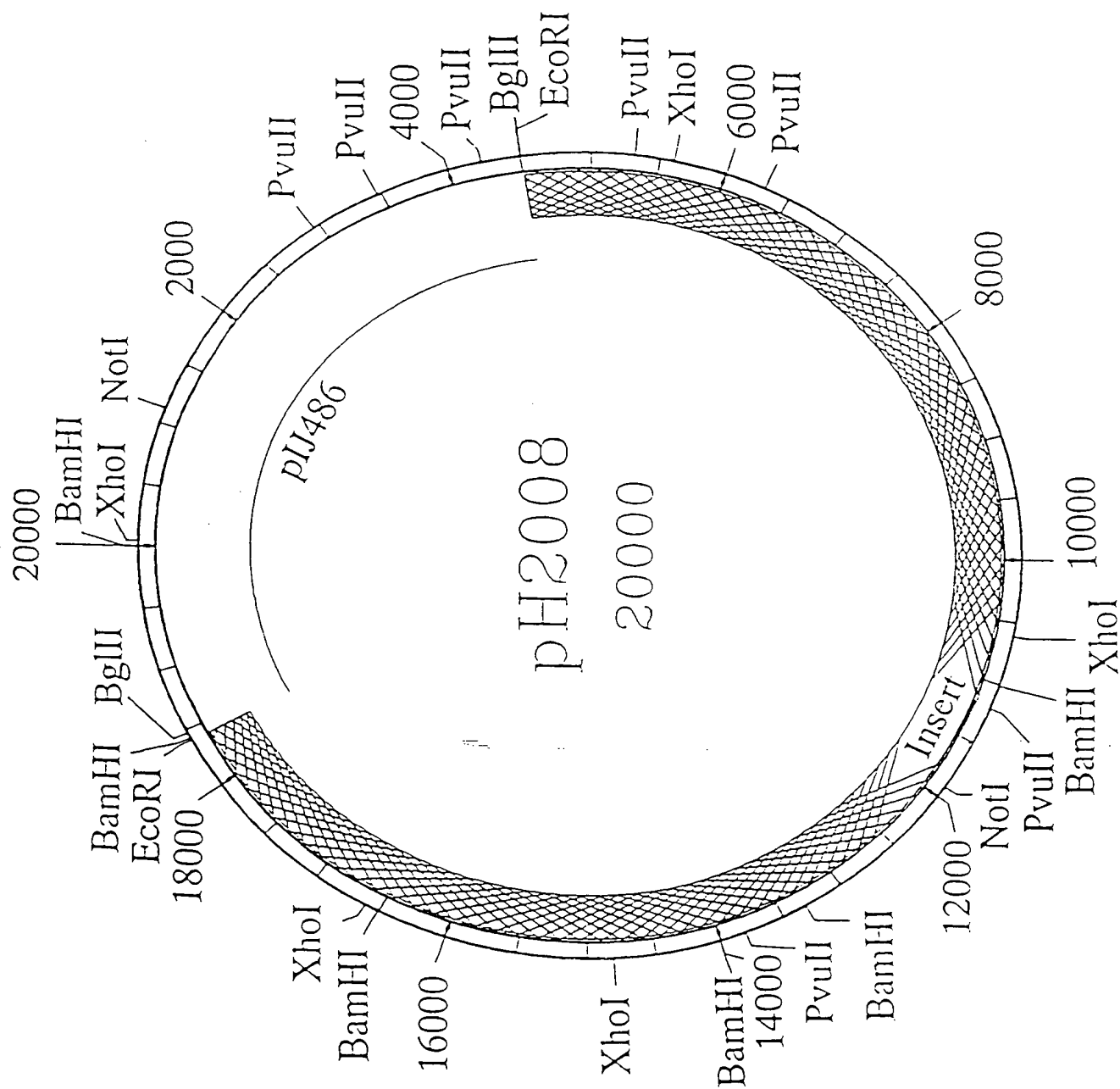


FIG. 4

5/9

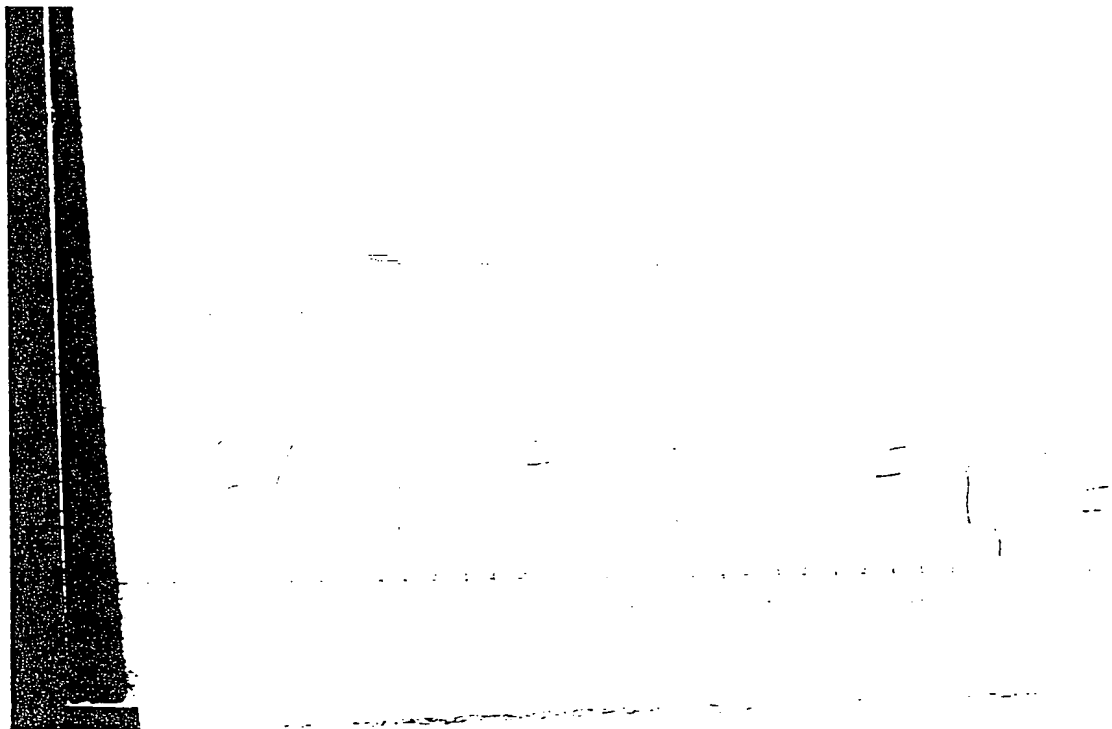
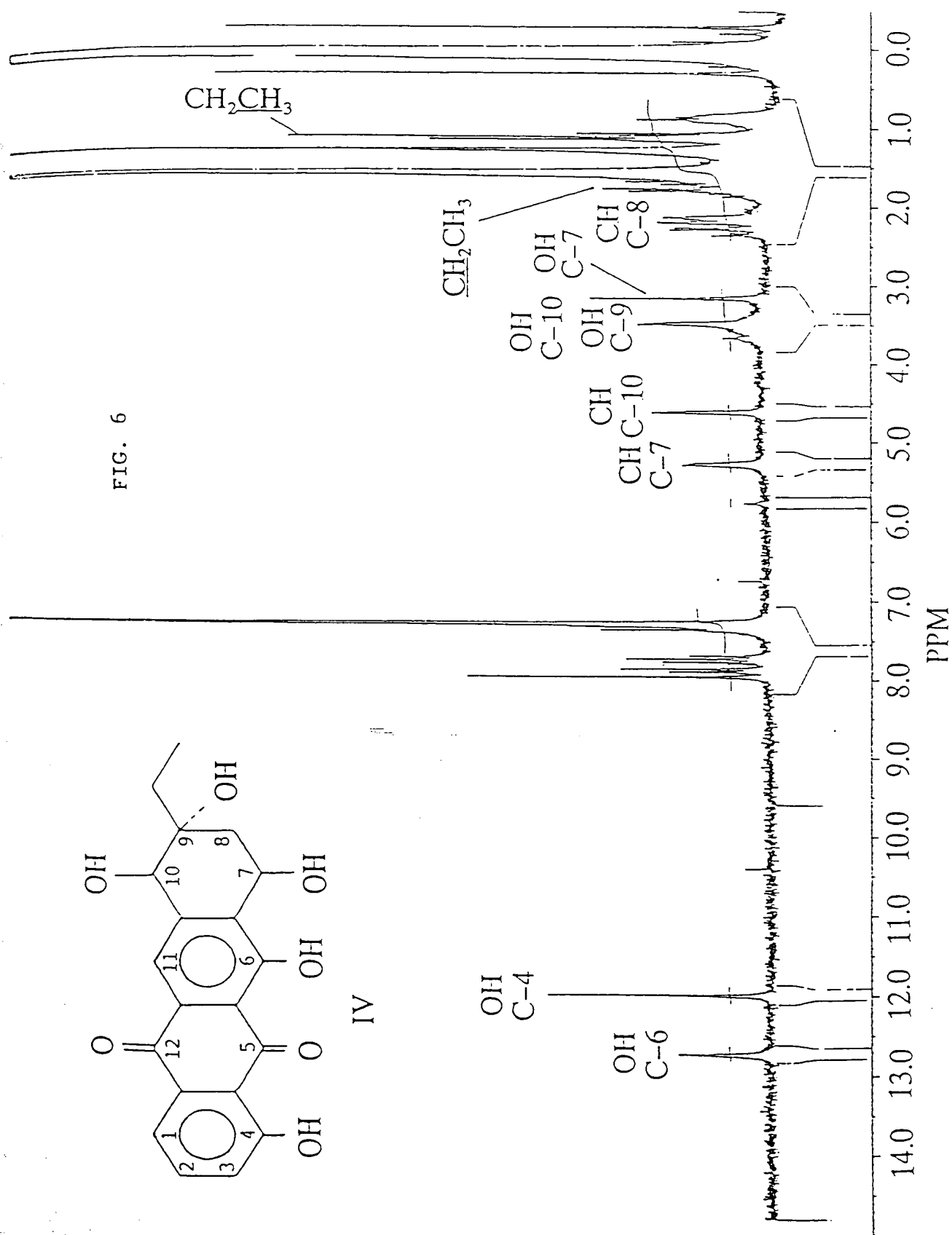
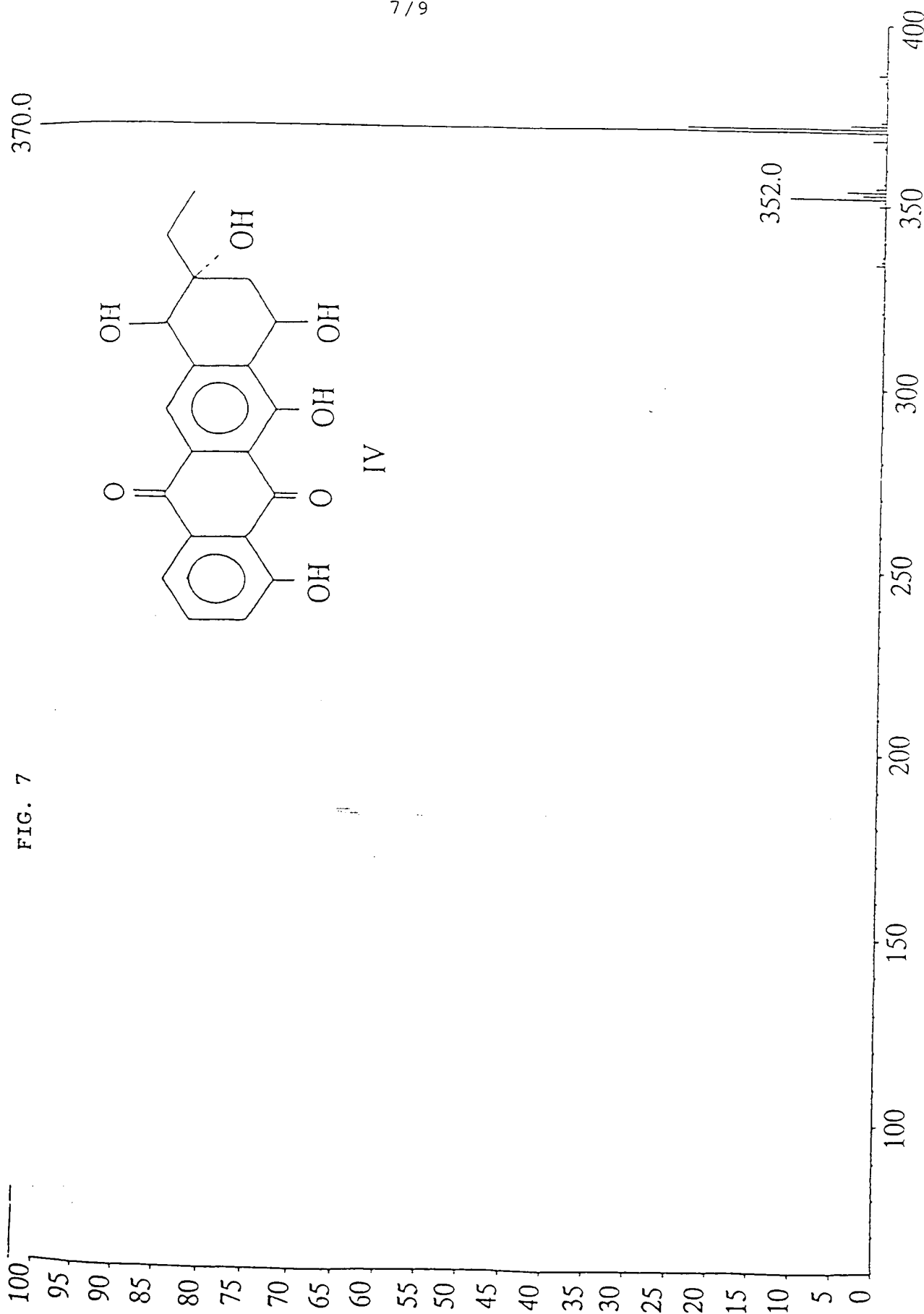


FIG. 5

FIG. 6



7/9



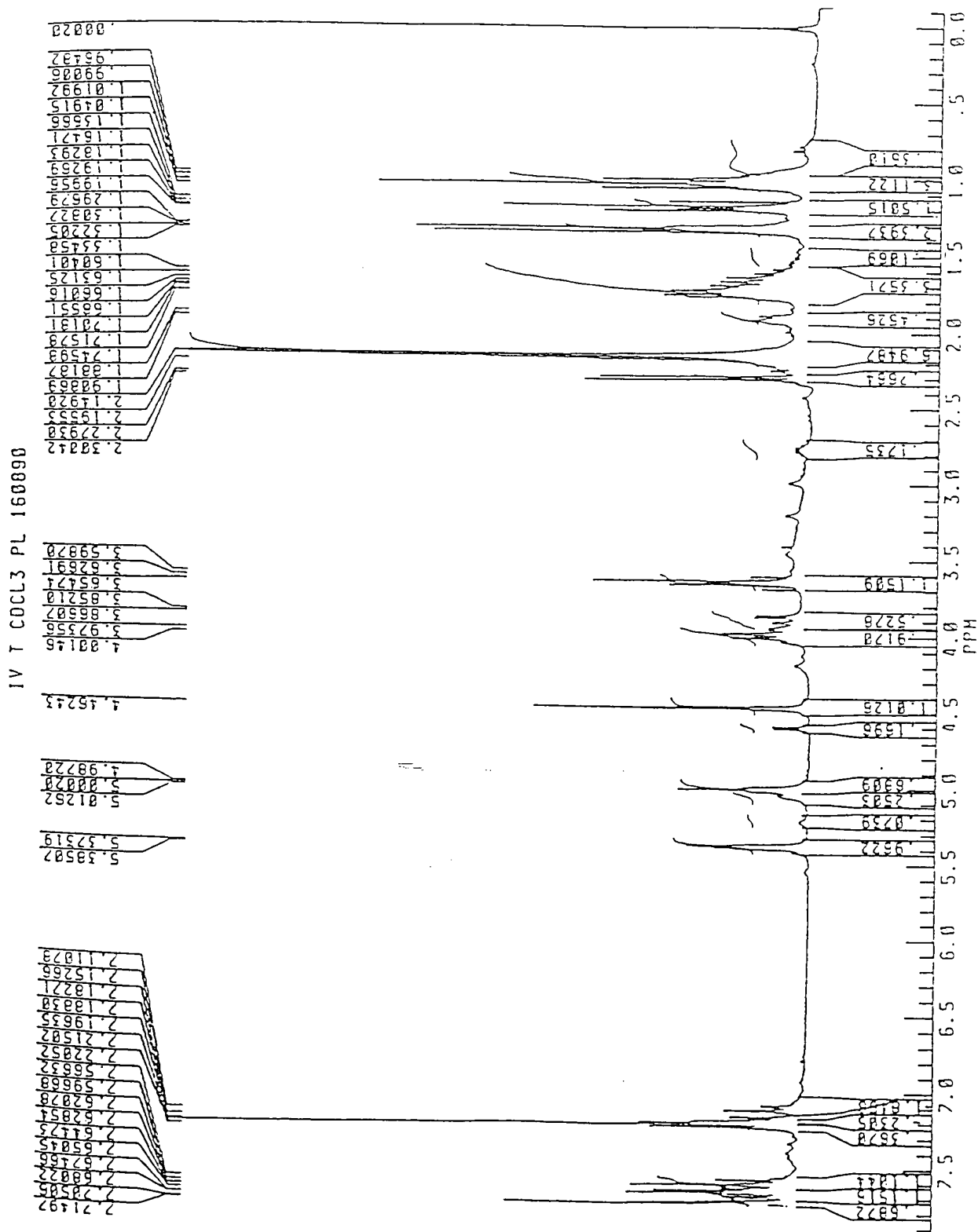
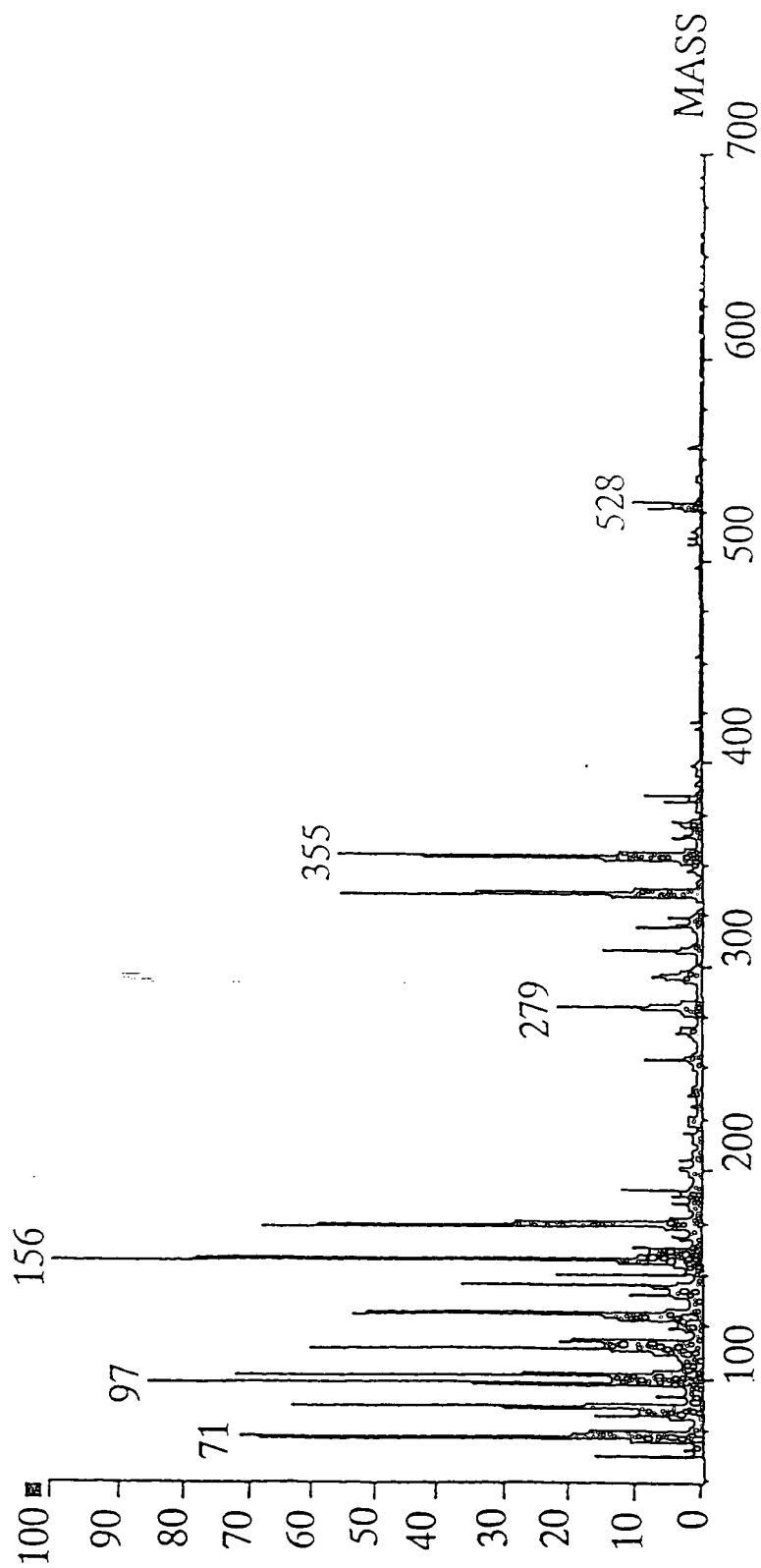


FIG. 8

9/9

FIG. 9



## INTERNATIONAL SEARCH REPORT

International Application No PCT/FI 92/00084

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) <sup>9</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC5: C 12 N 15/31, C 12 P 19/56		
II. FIELDS SEARCHED		
Minimum Documentation Searched		
Classification System	Classification Symbols	
IPC5	C 12 N; C 12 P; C 07 K	
Documentation Searched other than Minimum Documentation to the extent that such Documents are Included in Fields Searched <sup>8</sup>		
SE,DK,FI,NO classes as above		
III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup>		
Category *	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
Y	EP, A2, 0131181 (HOECHST AKTIENGESELLSCHAFT) 16 January 1985, see the whole document --	1,4,11, 13,14
Y	EP, A1, 0050724 (F. HOFFMANN-LA ROCHE & CO) 5 May 1982, see the whole document --	1,4,11, 13,14
Y	PROC.NATL.ACAD.SCI., Vol. 86, May 1989 Kim J. Stutzman-Engwall et al: "Multigene families for anthracycline antibiotic production in Streptomyces peucetius", pp 3135-3139 --	1,4,11, 13,14
Y	NATURE, Vol. 325, February 1987 F. Malpartida et al: "Homology between Streptomyces genes coding for synthesis of different polyketides used to clone antibiotic biosynthetic genes", pp 818-821 --	1,4,11, 13,14
<p>* Special categories of cited documents:<sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
29th June 1992	1992 -07- 0 2	
International Searching Authority	Signature of Authorized Officer	
SWEDISH PATENT OFFICE	<i>Carolina Palmcrantz</i> Mikael G:son Bergstrand	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	NATURE, Vol. 314, April 1985 D.A. Hopwood et al: "Production of hybrid antibiotics by genetic engineering", pp 642-644 --	1,4,11, 13,14
Y	BIOTECHNOLOGY, September 1984 Christopher R. Bailey et al: "Cloning a streptomyces clavuligerus genetic locus involved in clavulanic acid biosynthesis", pp 808-811 --	1,4,11, 13,14
A	EP, A2, 0299350 (BEHRINGWERKE AKTIENGESELLSCHAFT) 18 January 1989, see the whole document --	1-14
A	EP, A2, 0298385 (BEHRINGWERKE AKTIENGESELLSCHAFT) 11 January 1989, see the whole document -- -----	1-14



ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO. PCT/FI 92/00084

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the Swedish Patent Office EDP file on 29/05/92  
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A2- 0131181	85-01-16	AU-B-	578615	88-11-03
		AU-D-	2981284	85-01-03
		CA-A-	1244364	88-11-08
		DE-A-	3323025	85-01-10
		JP-A-	60036437	85-02-25
		US-A-	4713371	87-12-15
EP-A1- 0050724	82-05-05	AT-E-T-	11142	85-01-15
		JP-A-	57099200	82-06-19
		US-A-	4383037	83-05-10
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		DE-A-	3722698	89-01-19
		JP-A-	1029338	89-01-31
		US-A-	4914191	90-04-03